GENETICS

Genetic Information and the Control of Protein Structure and Function

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First Conference on Genetics

THE JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

DURING THE PAST fifteen years the Josiah Macy, Jr. Foundation has organized more than twenty conference groups, each group meeting for at least two days annually over a period of five or more years Each meeting is limited to twenty-five participants (members and guests), selected to represent a multidiscipline approach to some urgent problem in the field of medicine and health The goal of this conference program is the promotion of communication, the exchange of ideas, and the stimulation of creativity among the participants The purpose of the publication of the Transactions of the meetings is to share, as far as possible, the conference process with a larger audience than could participate personally in the discussions

These conferences provide an opportunity for informal give and take among the participants To further this purpose, the number of presentations planned for each day is generally restricted to one or two The member, or guest, selected to give such a presentation is requested not to "read a paper," but rather to highlight, in an informal manner, some of the more interesting aspects of his or her research, with the expectation that there will be frequent interruptions by participants in the form of questions, criticism, or comment Such interruptions during the course of a presentation are encouraged and form an essential part of the "group interchange"

The conference program has always been viewed by the Foundation as an experiment in communication in which there is room for improvement and need for frequent reappraisal. Sufficient experience has already been gained to justify the conclusion that this type of conference is an effective way of improving understanding among scientists in medicine and allied disciplines, of broadening perspectives, of changing attitudes and of overcoming prejudices The further conclusion has been reached, as the result of this experiment, that a major obstruction to understanding among scientists lies in the resistance of human attitudes to change, rather than in difficulties of technical comprehension Less extensive experience with non-scientists has indicated that the effectiveness of this type of conference is not limited to groups of scientists, but will function in any group meeting where more effective

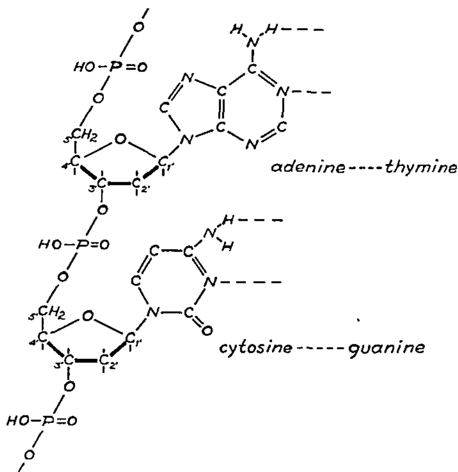


FIGURE 1 The covalent structure of DNA is represented by solid lines The dashed lines represent hydrogen bonds.

RNA, which contains ribose, there was much speculation that unusual linkages existed, but even in that instance, there seem to be only 3'-5'-linkages

I might say something here about the sequence of bases, on which subject there is almost no information. We do say that all sequences are possible, but that is not a strong statement. We might say that they occur at random, but that might be quite false, especially if coding is considered There is no reason to believe that all sequences are equally probable

Fremont-Smith: Do you mean by this that there is probably a regular but not yet defined sequence?

Watson: I certainly believe that there are many possible sequences. Let's say there are a large number of DNA molecules and they are responsible for different genetic functions. I believe that their sequence is the controlling difference, *i.e.*, they differ only in sequence.

Fremont-Smith: Each one would have a different sequence, but a regular, systematic sequence for that particular one?

Watson: Yes, a definite sequence for a definite function.

Fremont-Smith. Therefore, there are many DNA isomeric molecules?

Watson: Yes, in that sense. But with all the same backbone.

Anfinsen: Kornberg and his associates have described another technique for the determination of couplet pairing and of the sequence of couplets. They set up four different reaction vessels, each one containing, in addition to the condensing enzyme, a DNA primer and all four deoxynucleoside-5′-triphosphates. In addition, each tube contains one of the 5′-triphosphates in radioactive form. The newly formed polynucleotide is isolated and degraded with a specific diesterase which cleaves between the phosphorus atom and the 5′-hydroxyl group of deoxyribose to yield 3′-deoxyriboside phosphates. In this manner, radioactive phosphorus (present, for example, in the dinucleotide sequence -A-O-$\overset{*}{P}$-O-B-, where the radioactive P atom was originally part of the mononucleotide triphosphate, A-$\overset{*}{P}$-P-P) becomes part of the 3′-nucleoside phosphate $\overset{*}{P}$-O-B. In the example given here, it can be said that the two nucleotide residues, A and B, exist within the polynucleotide chain in the sequence A-B, rather than B-A. The results show, in general, that the bases are not distributed randomly along the chain, and that there are, definitely, some favored combinations.

Lederberg: Complementary couplets fit beautifully.

Anfinsen: They do fit beautifully, of course, in the double helix. These experiments, however, are more concerned with the order of bases *along* the chain rather than complementarity *between* two chains.

Watson: Have they used these different primers to show the complementary couplets?

Lederberg: Yes, and they have different neighbor compositions.

Watson: To recapitulate, the primary structure of DNA is a linear chain with no branches, usually at least 10,000 nucleotides in length, and with the four main bases occurring in a variety of combinations. The secondary structure of DNA is shown in Figure 2.

There are two intertwined chains with the sugar-phosphate backbone on the outside and the purine and pyrimidine bases on the inside. The purine and pyrimidine bases, which are relatively flat structures, are perpendicular to the long axis.

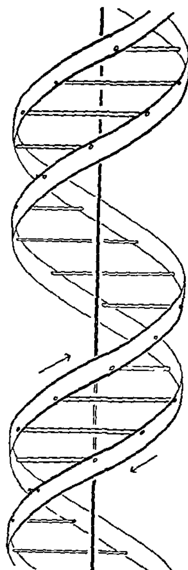


FIGURE 2. Double helical structure of DNA. Reprinted, by permission, from Watson, J D, and Crick, F H C. Genetical implications of the structure of deoxyribonucleic acid *Nature, London* 171, *964-967 (1953).*

The evidence favoring this structure is several-fold The first is x-ray evidence obtained from DNA fibers by Wilkins and Franklin (15,44). Within the crystallographic repeat unit which their x-ray pictures picked up, there was more than one chain. At first, it was im-

possible to tell whether there were two or three chains, but there were certainly more than one.

Several facts were considered in building this multichain structure. The most important was the observation that DNA from a single species gave a very regular diffraction photograph despite the fact that there were believed to be many different isomers of DNA present The most plausible solution was to construct a molecule with the sugar-phosphate backbone—that is, the part of the molecule which is regular—on the outside, so that all neighbor contacts occurred at the sugar-phosphate backbone surfaces Then, it became a question of trying to put several chains with irregular sequences together in a regular way It was necessary to decide whether to make a model with two or three chains Francis Crick and I put most of our effort into making a model with two, because it was the simpler one, and also because geneticists would prefer two, but the latter is a poor reason

From the earlier crude data, if the size of the unit cell and the fact that a nucleotide occurs every 3 4 Å were considered, three chains were obtained, although this was done under the assumption that there was no water in the DNA fiber However, Francis Crick and I knew that there is considerable water in the structure, but it was an unknown amount and very difficult to measure

Fremont-Smith· How did you know that?

Watson. We knew that very good diffraction photographs are obtained if water is present, but in photographs of dried fibers, essentially no structure is shown

Fremont-Smith So there had to be water present?

Watson Yes, and the question was how much This was never determined well Only rather large samples can be measured On the very thin DNA fiber, where good x-ray work is done, accurate measurement is impossible, but we guessed that 50 to 60 per cent of the volume might be occupied by water and, allowing this, there were two chains

Fremont-Smith: What is the factor that determines the tightness of the helix?

Watson· The sizes and shapes of the atoms.

Fremont-Smith: Is it the electrical forces between the atoms or between the chains? Why don't they come right up snug and attach, or why don't they separate farther apart?

Watson: Do you mean, why is the diameter around 20 Å?

Fremont-Smith Yes

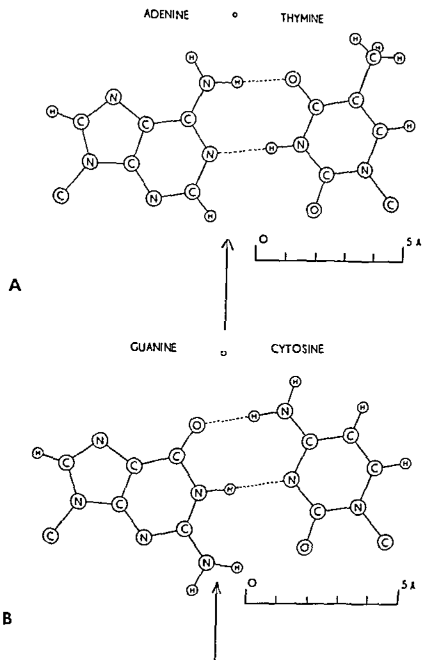


FIGURE 3 (*A*) Pairing of adenine and thymine. Hydrogen bonds are shown dotted One carbon atom of each sugar is shown (*B*) Pairing of guanine and cytosine

Watson: This is determined by the size of the bases.

Fremont-Smith: They do touch?

Watson: They are hydrogen-bonded to each other. This is shown in Figure 3. The diameter of the double helix is approximately 20 Å, with the bases stacked in the center That there are 10 residues in one turn of the helix comes from the fact that the bases are hydrophobic, or at least their flat surfaces are, and they don't like to be next to water but to each other So one starts out with a series of bases which stack over themselves as much as possible. The best way of doing this is to make one turn every ten nucleotides. It might be done with eight, but that would begin to be slightly uncomfortable It couldn't be done with twenty, because the sugars would interfere

The belief that this is the optimal configuration for most forms of the polynucleotide backbone is reinforced by the work of Rich and his group, who found that all the synthetic polynucleotides have a pitch of this order of magnitude (30); that is, they go round once each in 30 to 40 Å One would never expect to find them going around once in 50 Å

To return to the question of hydrogen-bonding, as early as 1949, Gulland and Jordan (18) obtained evidence that the bases of DNA are hydrogen-bonded They added either acid or alkali to DNA solutions With non-hydrogen-bonded bases, it might be expected that a titration curve similar to that of the free bases would be obtained, but this did not occur They found that they could add acid and get no reaction with the amino groups until the pH was approximately 3 Then, the DNA began to take up protons Similarly, on the alkaline side, protons were released only above pH 11 5 The authors correctly interpreted these findings as suggesting hydrogen bonds, but did not attempt a detailed structural interpretation

Fremont-Smith Would you describe a hydrogen bond for us?

Watson: I can give the simple explanation which was originally given to me On a keto oxygen, there is a slight negative charge, whereas, on hydroxyl or amino hydrogens, there is a weak positive charge A hydrogen bond is essentially a rather weak form of electrostatic bond Furthermore, there is bond energy only when the hydrogen points toward the electronegative atom

Fremont-Smith It takes energy to pull them apart?

Watson: Yes The amount of energy which it takes, in the case of an NH-O bond, is about 5 kcal This should be thought of in comparison with, say, a carbon-carbon bond, which is of the order of 50 to 100 kcal It is much weaker. On the other hand, it should also be compared with thermal energy (KT) which is 0 8 kcal.; so a bond with

KT energy will be formed easily and quickly, but it can also be broken relatively easily. A useful rule is that a stable structure will have as many hydrogen bonds as possible The difficulty in predicting nucleic acid and protein configurations is that hydrogen bonds also form with water

Fremont-Smith: Is the water-bonding weak?

Watson: No, it is fairly strong

Fremont-Smith: So it may replace these others at times?

Watson It is hard to predict whether water will hydrogen-bond to itself or to the bases. This can be decided only from experimental evidence In water, the bonds will be broken and re-formed constantly, so that only a certain percentage of them is present at a given time. With structures like the protein α-helix or DNA, a number of them are stabilized The best evidence comes from Doty's work on the synthetic polypeptides With these, the energy difference between forming bonds with water and forming them internally can be measured It is found to be an extremely small difference (9).

Fremont-Smith: Do these energies summate in a long chain and thus give stability?

Watson It is a form of summation

Fremont-Smith: It is exponential?

Watson. Yes So the fact that DNA is a very long molecule produces stability For example, in the case of synthetic polypeptides, if there is a very short chain length, a helix is not formed, whereas, if there is a long one, the helix is possible

I want to emphasize the fact that at present no one wants to predict which ones will occur It is necessary to obtain experimental evidence as to whether they occur internally or mixed In the case of DNA, the evidence came from titration In the case of protein, some evidence comes from deuterium exchange experiments, which suggest that a certain fraction of the hydrogen bonds cannot exchange.

Another important consideration is the correct tautomeric forms of the various DNA bases These were not commonly known in 1953. Francis Crick and I were very fortunate in sharing a room with the crystallographer, Jerry Donohue He told us that there was no doubt that guanine would be in the keto form, that adenine and cytosine would be in the amino form, and that thymine would have the keto configuration. When these tautomeric forms were used, the now familiar pairs of adenine-thymine and guanine-cytosine were quickly found.

I think that most people believe that when we constructed the DNA model, we took E Chargaff's data on equivalence of base pairs and tried

to build a structure with them. On the contrary, we initially tried to ignore these findings We thought they might have some functional significance, that is, something to do with DNA function The fact that these pairs existed in the DNA helix arose primarily from structural considerations. If adenine is put with thymine, the unit that results is exactly the same size and shape as the unit obtained from guanine and cytosine These are shown in Figure 3

Fremont-Smith. That was the basis of your making them equivalent?

Watson: Yes We wanted to build a regular structure in the center as well as on the outside

Fremont-Smith. Then, it only happened to fit in with Chargaff's data afterward?

Watson: Yes, and this was very pleasing

Glass: Sometimes, a third hydrogen bond is indicated in the guanine-cytosine pair

Watson: Yes When we put these base pairs together, the crystal structure of guanine was not known well It wasn't clear to us that if we formed two good hydrogen bonds, the third bond could be significantly strong The guanine structure still has not been determined accurately, but it now seems likely that there will be some bond energy at the third position Pauling and Corey (29) say there will be a third bond I now think there is, and the best evidence comes from the fact that DNA which contains more guanine and cytosine is more stable than DNA which contains more adenine and thymine

Fremont-Smith What is the evidence for showing the angle at which one of these atoms goes off from the molecule?

Watson, The only good evidence is crystallographic This has been done for adenine very accurately, and so we knew the exact bond angles Guanine was determined more roughly

Fremont-Smith. By x-ray diffraction of the crystal?

Watson Yes This is the only way for a molecule this complicated I don't know of anyone who would want to predict the exact bond angles and lengths It's sort of semitheory and semiempirical

Something we still don't know today is the proportion of the bases which are in the wrong tautomeric forms, that is, the enol or imino forms The chemical techniques are not sufficiently sensitive to detect the very small amounts which may be present

Anfinsen: Has anyone done deuterium exchange studies?

Watson: Yes.

Anfinsen. And do all the exchangeable hydrogens fall pretty much in one class of exchangeability?

Watson: Yes, they all exchange within a minute or so. There are a number of forms of DNA, particularly from mammals, in which a fifth base, 5-methylcytosine, is common The methyl group on the 5 position does not affect the hydrogen-bonding properties Moreover, the analytical data indicate that the amount of guanine equals the amount of cytosine plus 5-methylcytosine This is analytically convincing only in those cases where there is a large amount of the 5-methylcytosine.

Another exception to the common base pairs involves the DNA of the T-even bacteriophages, where cytosine is replaced by 5-hydroxymethylcytosine, to which glucose is attached. Again, the hydrogen-bonding ability is not affected

A situation which may involve an exception to the base-pairing rule involves the thymine-requiring strain of *Escherichia coli*, 15T⁻. When it is grown in the absence of thymine, methylaminopurine appears in the DNA (12) It is not clear what this is replacing, but it was at first thought that it appears in lieu of thymine

Smithies: Could Wilkins' experiments indicate that the crystal structure normally observed in x-ray pictures of DNA fibers is not related to the structure in the living organism? When this bacterium is grown in the presence of methylaminopurine, no crystalline form can be detected in its DNA, yet the bacterium still grows

Watson No, this is DNA from a thymine-requiring bacterium grown in the absence of thymine The bacteria died, and then a strange form of DNA was detected; this form has a great deal of substituted adenine on which a methyl group occurs

Smithies So, essentially, the animal does not survive when it has this type of DNA

Watson No, it does not, so I don't think it argues against the generality of double helical DNA

Lederberg: This DNA is deficient in thymine, in fact, or is that not clear?

Watson: This is not as clear as it should be. I have heard contradictory data from R. Markham and D. B. Dunn It was at first thought that the methylaminopurine was replacing thymine, but now the data supply just as strong evidence that it replaces adenine.

Davis In the case of a double helix with diaminopurine substituted for adenine, wouldn't the second amino group give exactly the same spatial relations as the possible third hydrogen bond of guanine-cytosine?

Watson: Yes

Davis: It should be quite analogous, and the ring distances would all

be the same. I wonder if there is any real question as to whether that third hydrogen bond contributes to stability, and whether diaminopurine couldn't be a way of testing that? It should be harder to melt the two apart

Lederberg: Is there any evidence of hydrogen-bonding between the free bases of nucleotides in aqueous solution?

Watson. Not that I know of Some chemists at the California Institute of Technology have tried to form mixed crystals of adenine and thymine I am told that one does exist, but it is not the thymine-adenine hydrogen-bonding found in DNA Needless to say, they have tried to see whether they could build a DNA structure based on this, and I am told they could not. There are a number of other possibilities for hydrogen-bonding adenine and thymine The reason they were not used in construction of DNA models was that analogous structures could not be made with guanine and cytosine

Steinberg: Have mixed crystals of guanine and cytosine been made?

Watson: Not to my knowledge

Steinberg: Did you imply that there was only one way in which these could be hydrogen-bonded?

Watson No, there is only one good way in which adenine and thymine and guanine and cytosine form equivalent pairs Donohue (8) has published a paper on the possible pairing arrangements of all the bases in all combinations There is a very large number of possibilities The restrictions come largely by putting a backbone on them and seeing how they fit together

We might now discuss the molecular weight of DNA in aqueous solutions For a while there was a rule that the molecular weight increased by about 1 million per year As isolation procedures improved, the figure grew larger and larger In 1945 or 1946, it was about 1 million By 1953, it had risen to about 6 or 7 million This increase seemed to slow down slightly, so that in 1958 *E. coli* DNA was about ten million Phage DNA, however, was coming out at 14 million

These data were obtained primarily by light-scattering As long as the DNA molecule was thought to be relatively small, this method seemed valid, but at molecular weights of about 10 million there are ambiguities in interpretation

More recently there has been used instead a combination of sedimentation and viscosity data It is not easy to measure the diffusion of DNA, so the conventional way of measuring the molecular weight by sedimentation and diffusion has never been done accurately Doty and his group (10) have measured the sedimentation constant and viscosi-

ties on a series of degraded DNA samples for which the molecular weight had been obtained by light-scattering. They obtained the following two relations: $S = 0.063M^{.37}$

$$[\eta] = 1.45 \times 10^{-6} M^{1.12}$$

S is the Svedberg Constant; M, the molecular weight, and $[\eta]$, the intrinsic viscosity. A sedimentation constant of 20 corresponds, upon extrapolating their data, to a molecular weight of 6 to 7 million, and a sedimentation constant of 30 corresponds to a molecular weight of 12 to 16 million.

Levinthal: However, it should be kept in mind that all of the experimental points on that curve are obtained for rather low molecular weight values

Watson: Yes, that is right That is because there are very great difficulties in doing light-scattering when the molecule gets very big But the relation holds very well at lower molecular weights. What has happened over the past 2 or 3 years is that now almost all sedimentation constants are above 20

Levinthal: However, in a similar analysis carried out by Butler, *et al.* (3), DNA's from various sources did not satisfy this relationship between sedimentation constant and molecular weight, although material from one source degraded to different extents did suffice.

Smithies. What has been done to exclude the possibility of aggregation?

Watson. The possibility that today's DNA molecular weights (10-14 x 10^6) result from aggregation of smaller DNA molecules is difficult to disprove. Against this idea is the homogeneity in sedimentation behavior of our better DNA preparations and the observations that changes in ionic strength or additions of proteolytic enzymes, chelating agents, or hydrogen-bond breaking reagents (*e.g.*, 8 M urea at room temperature) do not alter the sedimentation constant.

Smithies: Could there be breaks in the strands which occur at different points?

Watson: This sounds like the suggestion that was made some years ago by Dekker and Schachman (7), that DNA molecules contain non-opposing breaks in the two chains. The evidence against that comes from experiments in which the hydrogen bonds are destroyed (denaturation) without a decrease in molecular weight (11). Denaturation can be achieved in several ways. One is to lower the pH. This is not a good way, for if the pH is too low, purines break off and apurinic acid is formed. A better way is to heat DNA. If most DNA samples

are heated to 90°C., the hydrogen bonds break and the polynucleotide chains assume more random collapsed configurations

There are several criteria that have been used for detection of denatured DNA molecules They are all very empirical One is the UV absorption at 260 mμ The absorption of the native molecule is 20 to 30 per cent less (hypochromic effect) than the denatured. The reason why the UV absorption is less in the native molecule than in the denatured is not well understood.

The second approach is to measure the optical rotation In the native molecule, there is considerable optical rotation, but when it is denatured, the rotation decreases A third criterion of whether the molecule is in the native state is the reaction with formaldehyde Denatured DNA will easily react but native DNA will not do so at room temperature.

Denatured DNA solutions have very low viscosities The change is very striking, say, from an intrinsic viscosity value of 100 dl/gm down to 2 dl/gm Electron microscopic examination of such denatured solutions shows puddles of collapsed DNA This seems to be an all-or-none phenomenon, at least, as measured by the microscopists There are either long intact molecules or puddles

Fremont-Smith. Has a variety of forms of denaturation been used? What is the effect of urea?

Watson: It aids the denaturation Addition of 8 M urea will not cause denaturation at room temperature in tenth molar salt solutions, however, it lowers the temperature at which denaturation occurs

Fremont-Smith: Are there no significant variations by using different denaturing agents?

Watson. Not as shown by loss of viscosity, but this is a rather crude criterion A number of factors can be measured What was first noticed was the loss of viscosity Sedimentation can also be measured, but this is not a very useful criterion It might be expected that when the very long, thin DNA molecule collapses, it would move faster; the fact is that the sedimentation constant hardly changes.

Measuring viscosity requires a purified DNA preparation and is time-consuming The easiest criterion which always parallels this fall of viscosity is a rise in UV absorption at 260 mμ The following type of experiment is easily performed

A DNA solution is placed in a spectrophotometer, where the UV absorption can be read while the temperature is being increased The temperature is increased in increments of approximately 10 degrees, following which there is a 15-minute period for equilibration. The UV absorption is measured and the temperature increased again Marmur

and Doty (25) found that at first the absorption is constant, and then, depending on the DNA sample, it suddenly increases to a new level (Figure 4). The sharpest break known occurs in T4 DNA, where the break is at 85°C. and the increase in UV absorption is 42 per cent.

The temperature at which 50 per cent of the UV increase occurs is called the melting-out temperature, and this is dependent upon the salt environment Generally, 0.15 M NaCl is used If a lower salt concentration is employed, the melting-out temperature is shifted downward, because of a stronger repulsion between neighboring phosphate groups NaCl concentrations higher than 0 15 M do not strongly affect the melting-out temperature; however, it is very useful to add a chelating agent, such as citrate, to avoid complications from the presence of metals

If material which is heated beyond the melting-out temperature is allowed to cool, the UV absorption usually returns to a value about 20 per cent greater than the native sample. The denaturation is thus an irreversible phenomenon * When denatured DNA samples are reheated, the increase in absorption does not usually occur as sharply as before It seems that during the cooling process, some hydrogen bonds are re-formed, but not in the regular manner of the original molecule.

Fremont-Smith: Does the melting-out curve always rise so sharply?

Watson: It depends on the sample

Fremont-Smith: If the temperature were raised very slowly, would it be possible to change the curve?

Watson No, it would still be sharp. At 60°C, there would never be melting-out, even if heat were applied for a very long time.

Fremont-Smith. But if the temperature were maintained just below the melting-out point, would there be some increase in absorption? In other words, is it possible to break some of the bonds and not all?

Watson: Probably The kinetics aren't worked out very well.

Lederberg: There is a time constant of some minutes there

Watson: The way the experiment has been done is to wait 15 minutes for equilibrium, but a wait of 3 days would probably produce some effect.

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*Recent experiments by J Marmur and D Lane change radically current ideas about DNA denaturation. Under properly controlled conditions, in particular, high salt concentrations and slow recooling, there is significant renaturation of thermally denatured microbial DNA This is shown both by the reformation of biologically active transforming principle and by a variety of physical chemical measurements See: Strand separations and specific recombination in deoxyribonucleic acids biological studies *Proc Nat Acad. Science* 46, 453 (1960), and also P Doty, J Marmur, J Eigner, and K Schildkraut: Strand separation and specific recombination in deoxyribonucleic acids physical chemical studies *Proc. Nat Acad. Science* 46, 461 (1960)

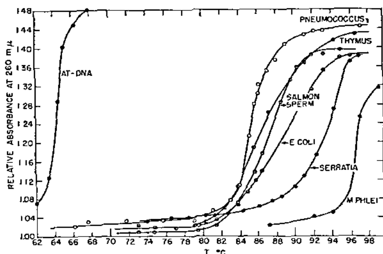


FIGURE 4 Variation in relative absorbance (2600 Å) as a function of the temperature of the DNA solution for various samples Reprinted, by permission, from Marmur, J , and Doty, P Heterogeneity in deoxyribonucleic acids *Nature, London* 183, *1427-1429 (1959)*

Fremont-Smith· Would that possibly give some clues as to structure?

Watson I'm not sure what it would tell; this is why the experiment has not been done But what the heating curves do tell, quite clearly, is that DNA from bacteria with high GC is more stable than DNA with high AT The stability of the pure AT polymer, prepared by Kornberg's group, is low The additional stability of GC DNA is very likely due to the third hydrogen bond present in the GC pair Dr Davis's suggestion, to use 2,6-diaminopurine in a synthetic polymer, would be useful to test this point.

Lederberg: What about the mixed melting point? Are two sharply distinct curves obtained if artificial mixtures of preparations with different melting-out points are used?

Watson. I am not familiar with that experiment It is possible to get an idea of the variation in content of GC and AT from the spread of the curve If there is a large variation with some molecules having more AT and others more GC, the melting is very spread out. T4 DNA, which is relatively homogeneous, gives a very sharp curve

I have recently done this experiment on DNA obtained from papilloma virus. Here again, when this DNA is heated, an increase in UV absorption occurs This DNA is shown by the conventional base analysis to have roughly equal proportions of all bases, and the melting

temperature is 89°C. But, unexpectedly, when the DNA is cooled, the relative absorption drops to about 1.1 rather than 1.2. When this denatured DNA is reheated again, a very sharp melting-out temperature of 89°C. results.

Levinthal: How high did you heat it?

Watson: To 98°C., which was above the point at which no further absorption increases are found.

Levinthal: How high did Doty have to go above the melting-out point before the effect was totally irreversible?

Watson: Experimentally, it is very difficult to go much above 96°C., so that means that for a bacterium which is high in GC, it is impossible to get far above the melting-out point. But for a bacterium high in AT, it is possible.

Lederberg: There are clear-cut irreversible effects with regard to hypochromicity, though, with all samples of DNA. There are lower temperatures at which the hypochromic effect is measured. At higher temperatures, there is a distinct diminution in optical absorption and in viscosity, which are essentially the same.

Levinthal. What is the sedimentation picture of the papilloma virus?

Watson. The purified DNA gives two very sharp peaks of sedimentation constant 28 S and 21 S.

Anfinsen: This could be dimer molecule.

Watson: By the Doty curve, this corresponds to molecular weights of 7 and 14 million. Robley Williams has recently taken very good electron micrographs of this virus, and Hugh Huxley also has taken some pictures of the papilloma sample which John Littlefield and I purified. The virus is spherical, with a diameter of 500 Å, and at high resolution it looks like a mulberry. There are apparently sixty protein subunits composing the outer shell.

The electron micrographs reveal that approximately 30 per cent of the particles are hollow and so many lack nucleic acid. If this is so, the published DNA content underestimates the content of the infectious particle which may be as high as 12 to 15 per cent. We might anticipate that the infectious virus particle having a molecular weight of about 50 million contains one DNA molecule of 6 to 7 million molecular weight.

Hoagland: Did you say what the relative concentrations of the two DNA components are?

Watson: About 30 per cent of the 28 S, and about 70 per cent of the other. Heating the DNA up to 90°C. and cooling it does not change

these relative amounts. I have also tried to change the salt concentration to see if I could change these amounts, but with no success

Levinthal: Have you done this in high salt?

Watson: About 4 M salt

Anfinsen May I take an emotional stand on something that has come up before? We generally discuss the physical properties of substances that have been isolated and analyzed in the laboratory, and very often there is a tendency to try to explain biological phenomena in terms of structures and molecular weights as they appear to us in an arbitrary solution. Actually, in many cases, these observations are only apparent and we have no idea whatsoever about the state of aggregation of molecules in their natural intracellular milieu In terms of extrapolating from what we measure to what happens biologically, we really are in the dark.

Watson. To return to the melting-out experiments with papilloma DNA, if the native molecules are heated to 95°C and then cooled to room temperature, the UV absorption returns to a relative value of 1 1 If the papilloma DNA is reheated, again a sharp melting-out temperature results

Atwood. After the cooling, did you see that any of the fibers had been restored, or are they all still puddles?

Watson. I haven't looked with the electron microscope

Atwood· Is it conceivable that this isn't a reversible process in the sense that fibers are restored, but rather, it may be that the kind of puddle you make happens to melt out in the same way as the fiber?

Watson: I doubt this I believe we are re-forming some native molecules I left the cooled sample undisturbed for 15 days, and the relative absorption fell further, to a 1 05 ratio

Atwood. Is the rate of re-forming concentration dependent?*

Watson: I have no data on that It is a very low concentration—about 10 μg DNA/ml

Cotterman Have you tried to cool it a second time?

Watson: I did one sample six times, back and forth During the last cycle, the melting temperature lowered by several degrees, but I think I was slowly breaking the polynucleotide backbone.

*Editor's Note Dr Atwood would like to add the following afterthought' to his remarks at the Conference

If the re-forming rate is independent of concentration, one might infer that complementary sequences occur on the same chain in reverse order, as Schwartz (33) once suggested This would permit the chain to pair with itself in antiparallel fashion Units of this type would show no change in molecular weight on denaturation, but would yield stable dimers on replication If the unit can be feasibly derived from the dimer, some peculiarities of papilloma DNA might be explained

Anfinsen: Would you make any guess as to why this reversibility is easier with some DNA's than with others? Do you think it is because of the arrangement of the bases?

Watson I don't know I think it's something which will be found out in the near future

Levinthal: It could very well be a matter of size since the probability of finding the right neighbor would be very much higher if there are only a few kinds of molecules

Watson: Yes, and the papilloma is certainly the most favorable case for recovery

There is one experiment in which the strands have been separated Meselson and Stahl (27) did this using cesium chloride gradients. The cesium chloride technique was developed (28) to answer the question, how do DNA molecules replicate? The obvious suggestion was that the two strands separate, each serving as a template for the formation of a new complementary strand (43) The problem was to distinguish new from old strands

The technique devised by Meselson and Stahl is very elegant They first tried to incorporate 5-bromouracil, a thymine analog, into DNA This DNA is heavier than normal DNA and can be separated from it in a density gradient. The difficulty is that it's hard to replace thymine by bromouracil uniformly Furthermore, this DNA is usually toxic Therefore, they switched to N^{15}, which did not seem to have any toxic effects

They grew bacteria (*E. coli*) in a medium which contained N^{15}, so that the nitrogen in the nucleic acid was heavy Then, the cells were transferred to a medium which contained N^{14}. The newly formed DNA was less dense than the DNA formed while the cells were growing in N^{15} Next, they devised a technique which would separate DNA containing N^{14} from DNA containing N^{15} This consisted of putting DNA into a centrifuge cell which contained a high concentration of cesium chloride. Cesium is a very heavy monovalent ion, and by using it, densities of 1 70, about the density of DNA, are easily obtained. When cesium chloride is put into an analytical ultracentrifuge cell, the cesium will begin to sediment and will set up a gradient which is a function of the initial concentration of cesium chloride and of the speed of the rotor. For example, the density at the top might be 1.65 while the density at the bottom is 1 75

If, in addition to the cesium chloride, DNA is added to the centrifuge cell, the DNA will form a band at the point at which its density equals the density of the cesium chloride. Let's say this is about 1 71 This is

a slow process, and it may require about 40 hours for effective equilibrium to be attained

When UV optics are used, the concentration of DNA in this band is easily measured, often revealing a Gaussian distribution, the width of which, under certain simplifying conditions, is proportional to the molecular weight of the DNA Both large and small molecules of the same density reach equilibrium at the same position However, the larger the molecule, the smaller its diffusion constant, and the less tendency to move away from the equilibrium position So, from the band width, the molecular weight can be estimated, if it is assumed that there is no preferential binding of cesium to DNA. The shape of the T4 DNA was perfectly Gaussian, and Meselson *et al* (28) reported that T4 DNA is homogeneous, with a molecular weight of 14×10^6 The error in their technique was such that the value might be between 12 and 16×10^6.

Davis: Would variations in the base ratios of individual DNA molecules affect the band width noticeably?

Watson Yes At first, this point was not considered Then, Meselson found, in comparing the T4 band with the band from calf thymus, that the calf thymus band was much wider and very skewed Moreover, when he removed DNA from a particular point in the calf thymus band and recentrifuged it, there resulted a narrower band This suggested density heterogeneity

This point has been carefully explored in the work of Rolfe and Meselson (31) and Sueoka, Marmur, and Doty (37), who studied DNA from bacteria with varying amounts of GC and AT and found that DNA which contains more GC is denser than DNA which contains high AT Consequently, if DNA from two different bacteria is used, two different bands may be found By studying DNA of varying base content, it is possible to arrive at a plot of the density versus the relative amount of GC (Figure 5)

The molar volumes of guanine and cytosine can be calculated and compared with those of adenine and thymine The GC pair has a smaller molar volume, accounting for at least one-third of the density difference Whether there is also some preferential binding of cesium by GC pairs is not known, in any case, we now accept as a fact the very good empirical relationship between the density of DNA and the base composition

Lederberg: Has anyone measured the partial molar volumes of free bases?

Watson: These have been calculated I don't think they have been measured

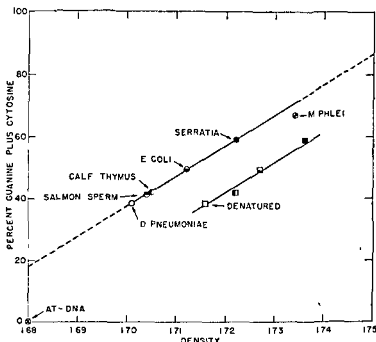


FIGURE 5 Relationship of density to the guanine-cytosine content of various samples of deoxyribonucleic acid Reprinted, by permission, from Sueoka, N, Marmur, J, and Doty, P Heterogeneity in deoxyribonucleic acids *Nature, London* 183, *1429-1431 (1959)*

Lederberg: It is very easy to do I think that with those values you should be able to separate the cesium-binding components from the partial molar volume components fairly easily. The reason why it may be important, of course, is that synthetic poly-AT does not fit on that curve.

Watson. Yes, the poly-AT is off.

Lederberg: I would like to know why You don't know whether *it* is off or everything else is off.

Watson: No. These relationships are highly empirical. Perhaps more interesting and certainly more unexpected was the finding that in bacteria with high AT there are no DNA molecules of a density found in the DNA from high GC bacteria. This suggests that there are no common DNA molecules between these extreme types of bacteria.

This was most surprising, because it was commonly assumed that within bacteria there would be many similar DNA molecules, controlling similar enzymes Hence, finding some DNA molecules with similar sequences might have been anticipated On the contrary, it looks as

though there will be no similar DNA molecules within these different types of bacteria

Estimates can be made of the band width spread resulting from density variation and that resulting from variations in molecular weight In the case of high molecular weights, the density heterogeneity may be quite important, for example, if the band width suggested a molecular weight of 20 million, density heterogeneity would become a serious consideration The crude molecular weight of *E coli* DNA based on the band width is 8 million, and by considering the melting-out behavior, from which one can independently estimate density heterogeneity, Sueoka *et al* (37) suggest that about 20 per cent of the spread in this DNA is caused by density variation The first results which Meselson got from salmon sperm DNA suggested a molecular weight of 4 million, whereas the physical measurements had indicated 8 million Certainly, most of this difference must have been due to density heterogeneity In the case of the more homogeneous bacterial DNA's, the band width is more closely determined by the molecular weight

To return to the N^{15} experiments, the experiment of Meselson and Stahl was to grow bacteria for several generations in N^{15}-containing medium so all the DNA was of the heavy type Then the bacteria were transferred to medium which contained only N^{14} and allowed to multiply for several generations After one generation of growth, all the DNA had an intermediate density as shown in Figure 6 This is called hybrid DNA, and it is composed of exactly half N^{14} and half N^{15} If the bacteria are allowed two generations of growth, pure N^{14} DNA is produced in addition to the hybrid DNA

One explanation for these results is shown in Figure 7 After one generation of growth, the DNA molecules have replicated so that each is a hybrid with one N^{15} strand and one N^{14} strand If each of these replicates another cycle, half the molecules will contain only N^{14} and half of them will be hybrid

There is one other possible interpretation of these experiments All that has been proved is that after one generation of growth, the DNA contains half N^{14} and half N^{15}. It is possible that this hybrid DNA is a dimer, composed of two DNA molecules aggregated end-to-end If, during replication, these molecules split crosswise instead of lengthwise, the results of this experiment would be the same as under the previous hypothesis

Atwood Could we have some clarification of this dimer hypothesis?

Watson: The theory is that the DNA molecule is really two double helices stuck together at their ends

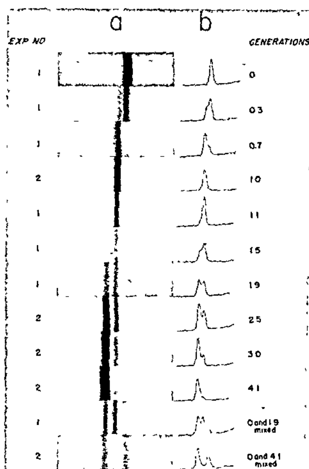


FIGURE 6 Ultraviolet absorption photographs showing DNA bands resulting from density-gradient centrifugation of lysates of bacteria sampled at various times after the addition of an excess of N^{14} substrates to a growing N^{15}-*labeled* culture Each photograph was taken after 20 hours of centrifugation at 44,770 *rpm* The density of the CsCl solution increased to the right. Regions of equal density occupy the same horizontal position on each photograph Reprinted, by permission, from Meselson, M , and Stahl, F W The replication of DNA in *Escherichia coli Proc Nat Acad Sc* 44, *671-682 (1958)*

Atwood: How do they replicate?

Watson. They make new copies by a two-stranded DNA model serving as a template for another two-stranded molecule, instead of a single strand serving as a template for a single strand

Meselson has done a control experiment,* to settle this point. He

* Personal communication.

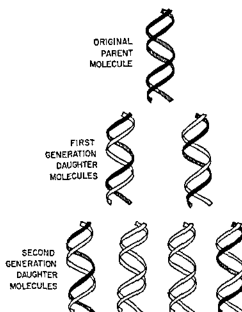


FIGURE 7 A mechanism for DNA duplication Each daughter molecule contains one of the parental chains (black) paired with one new chain (white) Upon continued duplication, the two original parent chains remain intact, so that there will always be found two molecules each with one parental chain Reprinted, by permission, from Meselson, M, and Stahl, F W The replication of DNA in *Escherichia coli* *Proc Nat Acad Sc* 44, *671-682 (1958)*

isolated hybrid DNA molecules and sonically vibrated them This transects the DNA molecules crosswise into double helical fragments If the lengthwise aggregation hypothesis is correct, this treatment should produce some fragments which have N^{14} and some which have pure N^{15}, whereas, in the other case, all fragments should have the hybrid density Meselson has calculated the results to be expected under the two different models, and he finds that the results rule out the end-to-end dimer possibility

Smithies: The sonic vibration experiment perhaps excludes end-to-end aggregation, but what about side-to-side?

Watson. Luzzati* claims that by using low-angle scattering, he can determine the mass per unit length of DNA in solution, and that his results are consistent with the double helix

*Unpublished data

Another interesting experiment concerns the denaturation of hybrid *E. coli* DNA. Native phage DNA forms a single band in the centrifuge When denatured, it becomes more dense and the band moves to a different position The explanation may be that in the hydrogen-bonded structure, the atoms are farther apart than when the hydrogen bonds are broken to give a random arrangement of the strands It is also possible that in the denatured material, there is a difference in cesium binding. These two possibilities have not yet been distinguished However, when the hybrid *E. coli* DNA was subjected to this procedure, two bands were present after the denaturation.

Lederberg. Do they change their absolute position?

Watson: Yes, they both change their absolute position. But the important point to keep in mind is that now there is one light band and one heavy band, that is, one which is N^{14} and one which is N^{15}. Their width now seems to be greater than it was before, and instead of being 8 million molecular weight, as in the native material, it looks as though it is now 4 million In one sense, this is a puzzling observation, for until this experiment the belief was that the DNA molecular weight is not lowered by denaturation

Levinthal The last conclusion about molecular weight is much weaker than the fact that there are two bands?

Watson: Yes It is very clear under these conditions that the *E coli* DNA has separated into two pieces Why they do not separate in the case of the T4 phage (or do separate in *E. coli*) is not known

I should add that the DNA replication experiment has been repeated with the alga *Chlamydomonas* (36). It is a slightly more complicated experiment because the cells do not divide regularly as they do in bacteria Instead, they divide in bursts Nevertheless, Sueoka observed hybrid DNA There also have been similar experiments at the California Institute of Technology by Edward Simon* using HeLa cells Simon can't easily use N^{15}, but he uses bromouracil instead It looks as though the results are compatible with the *E. coli* studies even though bromouracil complicates the experiment and there is just one DNA replication in the presence of bromouracil.

Ingram: It is very difficult to see how these strands pull apart with denaturation

Watson: All one can say is that they do.

Levinthal: It seems to me that intuition is not likely to be of much use in these problems The fact is that in the case of the artificial polynucleotides, the helix does unwind and wind up very readily. And if

*Personal communication.

the energies involved are calculated, there is nothing very surprising about this

Watson: I think our intuition in this is zero

Davis Couldn't the charge repulsions of phosphate be evoked to assist in the separation of the two strands, and if so, might it not be possible, by using a higher salt concentration and sufficient heat, to get them to go apart and cease being a double helix but still be tangled up in terms of the N^{14} and N^{15} distribution?

Smithies· Have you any thoughts on why the helix fails to re-form, as it does in the papilloma virus DNA after heat denaturation?

Watson· No It might be that in the case of the papilloma virus we had only two types of strands, and so the chance of finding the right one was good, whereas in the other cases that is not so

Lederberg· Crystallizing from an impure vs a pure solution

Watson: If it is true that it is possible to denature the transforming principle reversibly, as Julius Marmur* has said—and Meselson* also states that under the same conditions he gets the *E. coli* DNA to re-form—perhaps this will not be as rare as originally thought

Lederberg· We see reversible denaturation with respect to viscosity and hypochromicity at temperatures lower than the melting-out temperature, so we know these are situations where there is some residual structure which helps the double helix to re-form

Levinthal As one starts denaturing DNA, loops will presumably begin to form in which the hydrogen bonds between base pairs are destroyed But there will be a point at which the loops become so long that the probability of their finding their way back is zero or effectively zero There will also presumably be a stage at which such loops are so short that the probability of their finding their way back to the right position is essentially unity and one would expect to find all cases between these extremes But it has not yet been shown that the helix is re-formed under conditions where the strands are completely separated.

Fremont-Smith Has the effect of freezing been tried on DNA? You were speaking about the effect of water on the hydrogen-bonding

Watson Sometimes it is possible to freeze DNA and it doesn't do any obvious harm At other times we are not sure

Fremont-Smith Would it cause separation of the strands?

Watson. No

Lederberg: In Figure 6, is there a band between the hybrid and the parental DNA at 0 3 generations?

Watson No

* Personal communications

Lederberg. It looks so from Figure 6. Is that just an optical illusion? It would have a lot of bearing as to whether you replicate one molecule all the way or whether you can get partial substitution. Is the band width independent of the symmetry factor of the molecules?

Watson. It should be independent, because the symmetry factor enters into the rate at which the molecules move toward the equilibrium position in the same manner that it enters into their diffusion away from the equilibrium position.

Smithies: Does diffusion enter into the calculation?

Levinthal. Not directly The molecular weight enters into the equation The diffusion enters into it only in the rate at which equilibrium is reached. But, once equilibrium has been reached, the diffusion does not enter into it

Lederberg: How do you know that these are at equilibrium?

Watson: I suspect the bands in Figure 6 aren't at equilibrium. Meselson and Stahl were interested only in the relative centers of the peaks. The usual experiment is a one-day run, which is sufficient to establish the position of the bands, but for effective equilibrium, about 40 hours are necessary

Ingram · Could you comment on the density of the RNA? Is that at the bottom of the cell?

Watson. That is still very confusing. RNA seems to have an effective density of 2.04. So cesium chloride solutions cannot be used But Howard Dintzis* was able to band RNA in cesium formate. Whether this means a binding of cesium to RNA or not is not clear. Some measurements of the partial specific volume of RNA are considerably lower than those for DNA Some are 0.5, which would give a density of 2; so there may not be a specific binding. When data are obtained from the synthetic polynucleotides, we should be able to say something more useful on the subject.

Spiegelman: Have you ever run RNA in cesium chloride but with high levels of magnesium?

Watson. No

Spiegelman: We have, and it changes the banding position quite drastically.

Levinthal: I thought it would not band in cesium chloride.

Spiegelman: It doesn't band like DNA. I'm not talking about going to equilibrium but about letting the centrifuge run for a fixed amount of time to see what the effect is of changing the ionic environment

*Personal communication

Levinthal: Do you mean you are measuring the sedimentation velocity?

Spiegelman That's right, essentially One gets the impression that the magnesium is displacing some of the cesium

Watson. Yes, it could be.

Spiegelman· Does anyone here know the details of the report from Cavalieri, who claims that he can attack DNA with chymotrypsin?

Lederberg Among other things, he claimed (5) that the molecular weight of DNA could be halved by treatment with chymotrypsin, suggesting that smaller subunits may be held together by peptide linkages. However, he did not examine the separation of N^{14}- and N^{15}-labeled units from hybrid DNA Since Dr Levinthal's remarks require a thoroughgoing re-evaluation of the validity of molecular weights for DNA, it is not easy to take a firm position on reports like this

Anfinsen. What does Cavalieri postulate as to the nature of the chymotrypsin-sensitive cement? Does he think there is a protein involved?

Lederberg I don't know.

Watson· I will briefly summarize the discussion to this point by saying that the structure of DNA is known at the micro level, in the sense that we know that a small unit will look like the complementary double helix More difficulties arise at the macro level, particularly in connection with the structure of chromosomes and how DNA molecules are incorporated into them

I regard the general problem of DNA replication as solved Some might be more hesitant, but I think the evidence of the Meselson and Stahl experiments is conclusive

Ever since we have known what the structure of DNA is, there has always been doubt as to whether the strands could come apart easily The intuition of some of us was that it might be difficult, and of others that it was just hopeless Against this view that it was hopeless, there has always been Dr Levinthal He seems to be vindicated, in that the Meselson and Stahl experiment proves that the strands do come apart

Because we thought it was difficult for the strands to come apart, most of us have been loath to accept the idea that the chromosomes are one very large DNA molecule Rather, it seemed likely that chromosomes, in some way, were made up of DNA molecules held together by some invisible form of cement, which some would make protein and others would make magnesium ions, and so on. Generally supporting this idea, was the fact that when DNA was prepared, it seemed to be quite homogeneous For example, Meselson, using the cesium chloride method, analyzed the distribution of DNA across the band and found that it was

Gaussian Even if the possible complication of variation associated with base content were considered, the evidence looked fairly good

Recently, we have been shaken by the observations of Davison that DNA is rather fragile (6) Over the last couple of years, we have been so impressed by the stability of DNA with respect to temperature and so on, that we have neglected the fact that it should not be shaken even the slightest bit, because if it is shaken, it tends to break somewhere. The question now is, how much of the molecular weight distribution which we observe is artificial and how much exists in nature?

I don't think we know the answer, but we do know some experiments which must be done I think it would be appropriate if Dr Levinthal gave us some of his doubts concerning the homogeneity of DNA and some of the evidence on which they are based

Wagner· What is the present status of Plaut's work with *Crepis* (26)? Since his results are in disagreement with those of Taylor (39), I would like some comment on this disagreement

Levinthal. As far as I know, nothing further has been published and the technical difficulties which were raised to the original experiment still stand The measurements were made of the optical density on the film rather than of the number of grains or the number of tracks seen in the emulsion The statistics involved in the individual disintegration events were not taken into account in interpreting the data. But whether anything further has been done to eliminate these technical difficulties, I do not know

Wagner· Why don't you use Taylor's work (39) as an example of this type of duplication rather than just that of Meselson and Stahl? Do you have an objection to it?

Watson Taylor's results are compatible with those of Meselson and Stahl, but there is, as yet, no evidence that the units studied in Taylor's experiments are single strands of DNA You can easily imagine that the chromosomes had a polytenic structure, in fact, that they were made up of two DNA molecules and what was observed was the separation of whole DNA molecules The experiment shows that there are two units in the chromosome, but it does not show that these units are equivalent to DNA strands I think it is possible to build the chromosomal picture only after what is happening at the molecular level is known.

Fremont-Smith: Is part of the theoretical question when is a molecule a molecule?

Levinthal: I would like to answer that question in some detail, since it seems to me to be central to our discussions of the size of DNA. Let me start out by saying that my bias at the moment is that more is learned

about the nature of the chromosome by accepting as a tentative hypothesis the notion that the DNA of a chromosome is all one piece.

Atwood. Do you mean by a chromosome, that which is in a phage particle?

Levinthal: No, by chromosome, I mean the same thing that you do

Atwood Then, in that case, your view is quite untenable

Levinthal. I mean these little stainable objects in cells I realize that this is taking an extreme position, and I would like to take this extreme position to see how easy it is to eliminate

Atwood: It is an impossible position

Levinthal There are of course several ways in which one might hope to rule out such a hypothesis One is by considering the fact that whenever DNA is prepared free of contaminating material, it consists of pieces which are very much smaller than the amount in a chromosome This observation seemed quite meaningful since pieces which were more or less of the same size were obtained The estimates of the molecular weight of DNA have come from several sources The DNA of the phages T2 and T4 have been studied by means of sedimentation velocity, sedimentation equilibrium, and a nuclear emulsion autoradiographic technique The latter method, as used by Thomas and me (23), gave a molecular weight for one component which was of the order of 45 million, and in addition a number of smaller pieces were found Meselson, Stahl, and Vinograd (28), using the sedimentation equilibrium method, found that all of the DNA was of the same size and of the order of 12 million molecular weight, and the sedimentation velocity measurement gave a sedimentation constant of approximately 30 S units, all of the phage DNA seem to have this sedimentation constant Using the formula which was discussed earlier in the Conference, we find this is also equivalent to a molecular weight of about 12 million.

Thomas has suggested for some time that the lower values obtained in the ultracentrifuge might be a result of degradation of the DNA caused by velocity gradients in the fluid in which the material is suspended One of the difficulties however was in trying to understand why this shear degradation would be introduced in some experiments and not in others A possible answer to this question has been provided by Davison (6), who has shown that the velocity gradients produced when the material is introduced into the cell of the analytical centrifuge in the usual ways could be very high Experimentally he has shown that the shears in the hypodermic needle used to load the cell of the analytical centrifuge were in fact high enough to degrade DNA.

Because of the way in which the cells of the analytical centrifuge are

constructed, it has been necessary to introduce the material into the cell through a hypodermic needle, and the loading has generally been done with an ordinary tuberculin syringe. Davison injected the DNA into the centrifuge cell using as large a needle as possible and using a motor-driven screw to push the plunger When the injection was done very slowly, he found that the sedimentation constant which had previously been found to be about 30 S was now increased to approximately 60 S. Using the relationship that the sedimentation constant is proportional to the cube root of the molecular weight, this means an apparent increase in the molecular weight by something of the order of a factor 8 Davison also prepared some high-molecular-weight DNA that is free of protein by shocking the phage and then banding the DNA in cesium chloride, using the sedimentation equilibrium method This was done in a preparative rotor of the centrifuge so that the material could be introduced into the centrifuge cell without having to pass through a small hole The material which banded at the density corresponding to that of purified DNA was again used in the sedimentation velocity experiments, and again it was found to have a sedimentation constant of about 60 when introduced into the cell with low shear

Lederberg: How did he do the osmotic shock?

Levinthal: The osmotic shock was done by dumping 9.5 ml. of water into 0.5 ml of phage in saturated salt. How much shear is involved in this process is difficult to estimate.

Davison has also released the phage DNA by adding the phage to an alkaline buffer in the centrifuge cell, and the release occurred at the higher pH Again he found that the sedimentation constant was about 60 S

For the DNA prepared by either of these methods, the sedimentation constant could be reduced by subjecting the material to a high shear. The more rapidly the plunger of the syringe was pushed the lower was the observed sedimentation constant It is also interesting to note that the width of the sedimentation distribution was quite narrow in all cases. Although a sedimentation constant of about 30 is obtained if the material is injected with reasonable care, a value lower than this is obtained with further increase in the velocity gradient.

DNA can also be degraded by shaking a solution in a test tube or by putting a solution in a Waring Blendor. Cavalieri (4) has shown that if it is sprayed from an atomizer the molecular weights obtained are greatly reduced, to the order of 1 million molecular weight units or less

The question may then be asked, what kinds of bonds are being broken in such a velocity gradient? I think it is likely that in most cases

phosphate ester linkages along the backbone of the DNA are being ruptured At least, there is no evidence that one is breaking any other type of linkage. One reason for this is the fact that there does not seem to be any plateau beyond which further increase in the shear is ineffective

A more serious question is whether the forces that one would expect to be exerted on the DNA molecule in such velocity gradients are sufficiently great that it is reasonable to expect phosphate ester linkages to be broken

Unfortunately, a theoretical interpretation of this type of process can only be carried out in a very approximate form The detailed mechanics of the hydrodynamic interaction between a long chain and the fluid in which it is immersed is too difficult for exact solution Frenkel's analysis (17) gives some idea of what is occurring.

Although the detailed motion of a long-chain particle in a velocity gradient cannot be calculated, Frenkel did estimate the maximum tension to which such a chain would be subject This maximum tension will occur if the chain is stretched out and is lying at an angle of 45 degrees to the velocity gradient It is reasonable to expect that the velocity gradient itself will stretch out the chain in this form, but how long this process will take is difficult to estimate Some recent work in which the motion of nylon threads was followed in a velocity gradient indicates that the times might not be excessively long, however, this problem will doubtless require extensive further investigation

The important conclusion from these calculations is that the maximum force is proportional to the square of the length of the chain and the first power of the velocity gradient, and that the maximum tension will occur at the center of the chain This maximum tension can then be calculated for any given velocity gradient and this value compared with the maximum force which a covalent bond could stand

In order to produce DNA of molecular weight about 10 million, it would be necessary to break in half a piece of DNA which had a molecular weight of 20 million The formula shown in Figure 8 enables one to calculate that for a piece of DNA of molecular weight 20 million, suspended in a fluid with the viscosity of water, a velocity gradient of 5000 reciprocal seconds would produce a maximum force of about 2×10^{-4} dynes. This force is approximately equal to the maximum which could be exerted on a carbon-carbon single bond before it ruptured. Presumably, a phosphate ester bond would be somewhat weaker, although its exact strength is not known.

Since the force goes as a square of the length of the chain and since

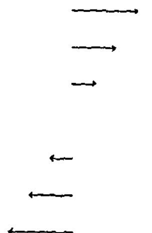


FIGURE 8 The maximum tension along the DNA length occurs when $\theta = 45°$ and this tension is equal to $\frac{1}{4}\pi\eta G L^2$ where η is the viscosity of the solution Forgacs and Mason (14) have carried out model experiments with threadlike particles in a velocity gradient and shown that such particles will in fact go through an extended configuration in which the tension is near maximum. Example Assume the viscosity η is about 10^{-2} poise, and that the maximum tension which the molecule can withstand without breaking is 2×10^{-4} dynes, which is a reasonable value for a covalent bond Then a velocity gradient of 20,000 sec^{-1} would break a 20 million molecular weight piece but not the 10 million molecular weight pieces produced

the velocity gradient cannot be increased in practice very much, it is not too surprising that a rather sharp distribution is produced.

I should add that this process of shear degradation has been studied with a number of polymers, and it has been shown that shears of the same order as are being considered here can, in fact, break a carbon-carbon bond.

Lederberg: But how do you expose every molecule in a solution to

these gradients? What is the maximum residue that could be unaffected?

Levinthal. Of the order of 5 per cent The densitometer tracing is flat at the region which corresponds to 60 S All these calculations are based on the assumption that the flow is laminar and smooth As the DNA goes into the needle, and as it comes out, there may well be turbulence Whether the breakage is involved at the laminar part of the flow or at the turbulent part of the flow is not clear

Atwood What is the form of the gradient in the laminar flow?

Levinthal The gradient is zero in the center and it increases linearly with the radius The piece of DNA of the phage which we observed in the nuclear emulsion experiments was of the order of 40 million molecular weight units and would have a contour length of the order of 25 μ The radius of a needle which is commonly used is of the order of 125 μ The velocity of the liquid at the wall is zero and the velocity of the liquid in the center is of the order of 10 meters/sec I don't know whether this can give you any feeling for the magnitude of the effect, but perhaps it makes it intuitively a little more reasonable. It is also obvious that one cannot really talk about the position of the molecule, since it is pulled all over the tube

Lederberg. But you get stream birefringence, for example?

Levinthal The time it takes the DNA to pass through the needle is of the order of a few milliseconds It is true that stream birefringence occurs, but does it start up in that time? Experiments of this kind have not been done

Davis: Could you give a figure for the standard deviation from the mean in an ordinary preparation? When you use the word "sharpness," what is the frame of reference?

Levinthal A figure can be given for the standard deviation of the sedimentation constant, but, of course, it is not known how that is related to the molecular weight

Lederberg Do you know whether the shearing effect is related to the length of the needle?

Levinthal: No

Lederberg. I wonder if the maximum effect isn't the transition between the barrel and the needle?

Levinthal It may be.

Lederberg. There is tremendous acceleration on the molecule which has just entered the laminar flow in the needle The part that is still in the barrel is moving very slowly, and the part which has entered the needle is moving very rapidly. There is a very large velocity gradient.

Levinthal: The velocity gradient, however, is still small compared to

the velocity gradient across the radius of the needle. It is necessary to keep in mind the fact that the liquid at the wall of the needle is stationary

Lederberg. But the liquid that is in the reservoir is essentially zero velocity compared to the rate at which it is moving through the needle at the transition zone. There is probably an even larger gradient there than in the needle.

Levinthal. If it were laminar flow, that would not be correct. If it were laminar flow throughout, the highest velocity gradient would occur at the narrowest place. But I agree completely that there is no *a priori* reason to assume it is laminar flow.

Davis: Is it definitely established that by progressive decreases in the amount of shear any desired sedimentation constant can be obtained, or are there quantum jumps that would suggest halving the molecule?

Levinthal: Davison's data so far do not answer this point But I don't think enough has been done. Suppose you start with a sedimentation constant of 60, if the molecular weight dropped in half, that is, if it went as $M^{1/3}$, the sedimentation constant would drop to only about 50. We think the data so far are certainly not good enough to determine that Whether they can be made good enough, I don't know.

Davis. Has Davison done the simple experiment of taking some of the DNA degraded to one extent by squirting it in fast, and to another extent by squirting it in slowly, and mixing them to make sure that there isn't an interaction that gives sharpening of the boundary?

Levinthal. Yes.

Davis: He can get two distinct boundaries?

Levinthal Yes, he can On the other hand, he has done one experiment which makes him think that the shearing effect is more a result of turbulence than of laminar flow in the needle If he slowly pushes some DNA which is not degraded into the centrifuge cell, and then squirts in some additional DNA rapidly, the whole becomes degraded. It is as though the turbulence in the bottom of the tube were responsible. If he prepares a DNA mixture in a test tube, half of which has been degraded and half of which has not, and inserts it into the cell slowly, the two bands can be seen very clearly. The experiments were also done as a function of concentration down to quite low concentrations and there was no obvious concentration effect.

Lederberg: What you are saying is that when there is a DNA solution at the bottom of a test tube, and water is squirted into that tube, the DNA is broken up in the bottom of the tube, so all these calcula-

tions about the forces inside the needle and laminar flow are completely irrelevant.

Levinthal: No, I don't think so Most of the experiments have been done with an analytical centrifuge, and those using this machine have been reasonably careful to avoid obvious turbulence. Ordinarily, when material is put into a centrifuge, it is done in such a way that the drops come out one at a time

Davis: Isn't it possible that there are weak spots in the DNA molecule?

Levinthal: None of this rules out the fact that there may be weak spots or even that there may be protein linkages which are breaking All the argument says is that one can equally well explain these data, and many others besides, without requiring that That is the essential point at the moment

What I am really suggesting is that all of these structures which have been called DNA molecules may have no real existence that even though it is possible to obtain a preparation which has a mean molecular weight of, let's say, 10 million, the ends of each molecule are not the same When starting with a very long structure, the position of the breaks may be essentially random, but a preparation which has a well-defined average size and a narrow distribution about this average size can still be obtained

One other point which I should like to make is that although everyone talks about that remarkable degree of homogeneity, there is no reason at all to know how remarkable this degree of homogeneity is or even how homogeneous it is These experiments have only been done with phage DNA so far, so that an enormous amount remains to be done The experiments which we did with T2 indicated that there was a large piece of DNA consisting of 40 per cent of the total, which on transfer to progeny phage seemed to carry half of the label it originally had As far as we know, this result is correct What caused us some concern was the fact that the sedimentation data always gave much smaller values for the molecular weight It may be, however, that the piece which we observed was artificially broken down from something larger in the intact virus But, the transfer data indicated that if it were a breakdown of a larger unit, the breakdown did not occur at random, but rather, it was the same piece breaking off in every phage particle

In general, it seems now that it is possible to say very little about the homogeneity of the molecular weights of DNA In order to measure these high molecular weights, it is necessary to have some experimental data and a formula And the formula implies a theory. Unfortunately,

there isn't any theory which tells us how these giant molecules would behave in solution Therefore, it is very difficult to obtain any meaningful interpretation of the hydrodynamic data. Light-scattering data do not help very much either for these extremely large molecules, which are so much larger than the wavelength of the light used. Under these conditions, none of the theories which have been worked out applies with any precision

Watson · Cesium chloride should work

Levinthal: There have been by now quite a large number of factors pointed out which could lead to an erroneous value of the molecular weight as obtained from the sedimentation equilibrium measurements. First, there is the question of possible entrapment of cesium chloride in the large molecule This is probably a very small effect A second difficulty in using the methods for molecular weight determinations has to do with the density heterogeneity which could be introduced because of the differences in the base ratio, because of the differences in small amounts of adsorbed protein, and because of the fact that any shear degradation which occurred prior to the measurement would affect the sedimentation equilibrium as much as any other measurement. On the other hand, most of the possible artifacts of this method would produce an artificially low molecular weight, so that if very high values are found by this method, they are probably believable.

To return to the question I raised earlier, I should like to ask if anyone has any convincing argument which says that all of the DNA of a chromosome is not in a single piece

Atwood Yes An inversion in a chromosome can be obtained, also, a ring chromosome will fall free when it divides. Suppose that you write the DNA structure, and then break two of the linkages and turn the segment around Will it still go all right? It is difficult to see how this would happen by breaking the 3,5 linkages

Lederberg: There are two antiparallel chains.

Atwood. But you couldn't invert a single strand of the DNA?

Levinthal. No

Atwood · What if you make a ring out of it? Can you make a ring that will come away free when it divides? This is a common property of chromosomes

Lederberg: There is no answer to that.

Atwood: Of course, it can be said that there is one spacer that handles that problem, and the rest of it is all DNA

Wagner: Use Taylor's model of the chromosome (38), where there are the little protein pieces.

Levinthal: The basic rationale for the Taylor (39) and the Freese models (16) was the observed existence of 7 or 10 million molecular weight DNA units If that is questioned now, the whole rationale of that kind of model must be questioned

Atwood: In some cases, a fine structure is visible in something that seems to be a representation of a chromosome, such as a salivary chromosome This fine structure suggests an alternating nucleic acid protein sort of sequence.

Levinthal: There are a number of answers to this For example, it is clear that in a chromosome which has dimensions of the order of a few microns, if the amount of DNA has a contour length of several cm, the DNA does not just stretch linearly from one end of the chromosome to the other, the DNA must be going back and forth some way However, it is not necessary to say that the chromosome is uniformly dense in DNA

Lederberg There is no evidence that the phosphate ester linkage is interrupted?

Levinthal: That is the question If there were a considerable amount of DNA in one band and only one strand going to the next band, it would not be possible to detect it with present methods

Atwood: The DNA content of certain salivary bands in *Rhynchosciara* is reported to increase enormously without any change in adjacent bands (1,13,32)

Marks: Are you suggesting that each chromosome corresponds to a single DNA molecule and in man, as an example, there are only 46 DNA molecules?

Levinthal: Perhaps twice that number I don't know what happens at the centromere

However, if the two chains of a very long piece of DNA are wound around each other, is there any way in which they can be unwound without getting into enormous difficulties? Or rather, what kind of difficulties could be expected other than the unpleasant complexity of the motion?

When the duplication of the phage T2 is considered, the apparent difficulty becomes immediately obvious The large piece of the phage DNA has a molecular weight of about 65 million and it duplicates in approximately 2 minutes This means that if it is to behave as a single piece, it must rotate with a speed of about 6000 rotations per minute. Furthermore, its contour length is about 25 μ, and there are some fifty pieces duplicating simultaneously in a cell which has dimensions of about 1 by 3 μ.

On the other hand, the diameter of one molecule is only about 20 Å units, and therefore a point on the circumference would only have to travel with a linear velocity of the order of 20 μ per minute, which is very small compared to the velocity of Brownian motion of the particles, so we can only conclude that the mechanics of the problem has to be calculated.

Unfortunately, attempts to understand the motion of a macromolecule in solution are complicated by the enormous mathematical difficulties involved in the hydrodynamic problem as well as the fact that our intuition does not apply to the type of motion involved. If I spin a piece of rubber tubing between my hands at one end, it is possible to make a fairly accurate prediction as to the general type of motion which the tube will undergo. In the case of rubber tubing, inertia and gravity play the dominant role in determining the motion, and it is for motions of this type that our intuition is of some use. In the case of a molecule like DNA, in which the length can be very great but the diameter is only about 20 Å, the motion which will take place in response to an applied torque is determined almost *entirely* by the viscous forces The resulting motion is of a type for which our intuition gives no appreciation or understanding

In order to carry out a model experiment with macroscopic objects, the problem must be scaled in such a way that the viscous forces remain the dominant ones This situation can be approximated by a very slow rotation of a thin plastic tube in a tank of highly viscous liquid like glycerin If the rotation is done sufficiently slowly, the tube, if it is initially curved, does not flail about as would happen if the motion were rapid, but rather, the tube rotates smoothly on its own axis as though it were an encased cable of the type used for transmitting the motion to a speedometer The reason for this on-axis rotation is that it allows the maximum rotation for the least expenditure of energy against the viscous drag.

The principle that an object in a viscous medium will respond to a force by that motion which will result in the least *dissipation of energy* against the viscous drag was enunciated by Jeffreys in 1921. To my knowledge, no general proof of it has yet been given. However, there is no question but that it applies in the special case being considered here The calculations of the amount of energy expended by such rotation and the torque necessary to produce it can only be carried out approximately, due to the uncertainties as to the effective radius of curvature and the unknown viscosity of the medium inside the cell in which the rotation is taking place. However, to a first approximation, one can make the cal-

culation by considering the entire molecule to be a long straight cylinder rotating at a constant rate in a liquid with a viscosity equal to that of water As long as the radius of curvature of the real molecule is large compared with its diameter and the medium in the cell does not form a gel, these assumptions are reasonably well satisfied Under these conditions, the torque required for the rotation is proportional to the rate of rotation measured in revolutions per second and also proportional to the length of the object which is being rotated. The rate of rotation is given by the total length of the molecule divided by the pitch of the spiral and the time required for the total rotation to occur Thus, the torque is proportional to the square of the length divided by the time Since the energy per turn is proportional to the torque and one turn is required to separate ten nucleotide pairs as the parent molecule duplicates, it is evident that the energy required to accomplish the rotation per nucleotide pair is proportional to the length of the total molecule divided by the time of duplication The equation (22) for this energy per revolution of the chain is

$$E = 16\,\pi^3 \frac{l^2}{P} \frac{\eta r^2}{T}$$

where P is the pitch of the helix (34 Å), r is the radius (10 Å), η is the viscosity of the medium *taken as 0.01 poise*, and T is the time of duplication of the entire DNA The units of E are ergs per revolution

I will assume that a DNA molecule must rotate one revolution for each turn of the parental spiral in order to duplicate itself and that this rotation is necessary for the separation of the two chains of the parental molecule Then we can ask how long a single molecule could be and still rotate in the way described here if the energy available for the rotation is about 8 kcal /mol This amount of energy is assumed to be available when the pyrophosphate is cleaved from the triphosphate precursors used as each new nucleotide is added in the formation of the new molecules By putting 8 kcal x 20 in the left-hand side of the equation given above, and 8 hours for T in the right-hand side, it is possible to solve the length l The result of this calculation is that a molecule of particle size of about 3×10^{10} molecular weight units could be rotated sufficiently rapidly to duplicate in about 8 hours Since this *is approximately the* amount of DNA in a single chromosome, we can conclude that even this enormous amount of DNA in one double helical chain could rotate as a single unit and that the rotation itself does not necessarily require the fragmentation into smaller pieces

Obviously, this argument does not in any way show that the DNA of a chromosome is all in one piece However, together with the result

obtained by Davison, it means that there is no compelling argument against the hypothesis that the single genetic map observed in a chromosome is a reflection of a single chemical entity. It is true that all of our intuitive feelings about molecules indicate that this preposterously long single molecule could not occur. However, some method of eliminating this hypothesis more rigorously should be sought.

Anfinsen: I'm also worried about the fact that, in building out a new chain, a large number of free mononucleotide molecules are converted to nucleotide *residues* in a DNA chain. The excluded water volume per nucleotide residue in such a chain is probably much greater than for a free nucleotide in solution due to the geometrical properties of the double helix There is, then, the heat of vaporization of the difference in water volume to take into account.

Levinthal· The energy source is presumably the triphosphate It is the splitting off of the pyrophosphate. Since you form new chemical bonds at the point where growth occurs, there will be, over very short distances at least, mechanical forces which bring the new nucleotides into their correct position. These mechanical forces could act to push the two old strands apart and thus cause the rotation of the remainder of the molecule in the same way that pulling the strands of a rope would cause it to rotate

Lederberg There is one trouble with that; that is, the specificity of the placement of the nucleotides depends on that force being very low, indeed, because it has to be comparable to hydrogen-bonding forces. It is these that determine whether you are going to put adenine and/or guanine into a particular place If it is necessary to unwind in the first place in order to get the nucleotide in, the kind of energy that is required for the splitting off of the phosphate cannot be expected, so there won't be that much difference between one and another

Levinthal I don't think that you can ask for a detailed chemical model of what would happen at the growing point, certainly not without knowing the detailed chemical reaction which is catalyzed by the polymerase molecule of Kornberg Basically this is the problem of how chemical energy is converted into mechanical energy in biological systems, and I don't think that we can answer the question here.

I don't mean in any of this discussion to say that there is any direct evidence which would indicate that all of the DNA in a chromosome is in one strand but most of the evidence to the contrary is very questionable. The reasons why people have thought of other models are now much less compelling than they were, and it is necessary to start looking at this possibility.

Another point I would like to mention has to do with the problem of pairing of chromosomes, of phage or bacteria or of higher organisms The specific factors of the DNA structure seem to be buried on the inside, so that one wonders how the specificity can be expressed I don't think that the fraction of the base pairs which are hydrogen-bonded in the DNA molecule within the cell is known It is perfectly possible to postulate the existence of loops in which the base pairs are not made and to imagine these loops as the regions of pairing specificity It is important at least to keep in mind the fact that the structure which the crystallographer sees is that of the bulk of molecule It is certainly not necessarily the structure of the entire molecule.

Spiegelman: How much longer is the DNA strand than the chromosome?

Levinthal: A factor of 10^4, let's say

Spiegelman In the sort of model you have mentioned, the DNA must be bunched in a very distorted way Whatever this distortion is, it can't be complete loops around the entire chromosome, in other words, in the distortion and bunching, some sort of relative geographical location must be maintained

Lederberg There is a lot of evidence for this Nuclei often form chromocenters so that the chromosomes are not altogether disarrayed in the interphase nucleus, also centromeres are likely to be located near one another This kind of relatively nonspecific association seems to be a good starting point for the very specific synaptic forces that develop later, however, a very high order of structure is not necessary in order to get this

Spiegelman: But you have the problem of packing a thread which is about ten times longer than the linear space available. It has to be bunched up somewhere

Lederberg Is there any evidence that double strands of DNA pair with other double strands?

Levinthal No.

Lederberg: Why should one think that synapsis involves this, when there are all kinds of gene products which may remain to some extent in association with the chromosome? I should think that pairing probably involved these products rather than the DNA itself Of course, somewhere, that DNA has had to open up in order to do anything to the cell

Levinthal I am only trying to see if the simplest model could operate. Is there any reason not to believe that pairing occurs between free DNA molecules?

Stern: If two DNA strands are paired, is there a random orientation of corresponding nucleotide pairs in the two strands relative to each other, or is there a preferred, fixed orientation? In other words, do the two strands turn specific pairing faces to each other?

Watson. The fact is that there is no reason why two DNA chains should even come together.

Stern: They do come together That is known.

Watson. But how close they are on the molecular level is not known.

Stern They are enclosed in some envelope. If this envelope must have a face, can this face be impressed on it by what is inside?

Watson: If an envelope is invented, it can be made any way that is desired

Stern But there must be an envelope, because you just said the DNA won't pair alone

Watson. I can think of no reason why double-stranded DNA would pair. It seems as though the phosphorus atoms would like to get as far apart as they could Two possibilities can be considered. First, should we think only in terms of double helical DNA, or aren't there some regions where it is spontaneously opening up, with free bases available for external pairing? I don't like this explanation

The other possibility is to invent protein, ribonucleic acid, or anything which might be a product of it, and say that this, in some way, brings the DNA helices together. As long as one is dealing with the chromosomes, where there are centromeres and so on, there is not much difficulty Difficulty does arise if the pneumococcal transformation is considered, especially in the case of free DNA which knows where to go A critical experiment (24) reports that the pneumococcus DNA molecule can be broken down into pieces one sixth the original size and still have these pair with host DNA. In such a case, it is difficult to rely on protein which might be still attached

Lederberg: The fragment might make some.

Watson: But this would not be a sort of structural protein, in the sense that it would lodge on the chromosome. Pure DNA means it would have no more than one per cent protein and one per cent protein is 100,000 molecular weight.

Davis: It has been done with much purer DNA than that.

Watson: It doesn't prove anything unless the protein content is very low combined with a very high specific activity of DNA In Hotchkiss's paper, I don't think this was so. One must admit the possibility that

there is a little protein coming along. However, if the DNA molecule can be broken up and the small pieces still function, I begin to believe that inventing a structural protein is of no advantage.

Sutton Even in the form of a helix, aren't the bases available in the large groove?

Lederberg: A structure that fills that condition has been built

Watson Three-stranded helices can be made on paper But I think they are very bad structures in that they are made only by putting guanine in the enol form I'm very loath to accept that

You can also try to build four-stranded helices. Leslie Orgel and I attempted to determine whether or not a two-stranded helix could make another two-stranded one It can't, if the restriction is maintained that the backbones are regular Drew Schwartz also has tried schemes, which involve two strands making two other strands But no one really likes these structures

Smithies: In a three-stranded structure, could the energy required for the keto-enol isomerization be supplied by the energy available as a result of forming the structure?

Watson No, because the energy difference between these two forms is at least 5 kcal and the net gain obtained by forming a hydrogen bond with a base instead of with water is probably less than 1 kcal

Stern Is anything known about the magnitude of the pairing forces of two double helices coming together or two chromosomes coming together? How do those forces compare to the bonds between successive nucleotide pairs? I would like to know, for instance, if, in these pairing forces, a twist were involved somewhere, would that be sufficient to break the bonds all across, so that opportunity for crossing-over would be provided?

Watson There is no reason why they should come together

Lederberg There is no reason for there being any forces at all

Stern: But they do come together That is known

Levinthal What we don't know is whether these forces are caused by interactions of DNA or interactions of chromosomes

Neel Dr. Stern, what is the evidence for a preferential point at which pairing begins in a chromosome pair?

Stern I don't know that there is very good evidence. The cytological evidence seems to indicate that pairing starts on the ends, but simultaneously it also starts somewhere along the middle, and then it seems to zip together from all these paired sections

Wagner. Don't the salivary gland chromosomes twist when they are paired?

Stern. Yes

Wagner They twist around one another, which, I suppose, is the reason you asked that question about the two faces. If one is coiled, the other will have to be coiled

Davis I would like to come back to the point that Dr Levinthal made about the possibility of regions where hydrogen-bonding does not take place I suppose he had in mind the possibility that the regions might form and then close in the course of time. Couldn't that be tested by the use of formaldehyde? Wouldn't you think that, once you had reacted with such a region, it would never rejoin, and in the course of time more and more formaldehyde would be taken up?

Levinthal· Yes, I would.

Lederberg· We ought not to preclude the possibility that there are denaturing agents acting on the chromosomes inside the cell There are all sorts of things that could be piling up locally in the cell that would diminish the hydrogen-bonding between segments, even if it is something not seen in aqueous solution outside.

I certainly wouldn't want to dismiss this kind of model for the interaction, but I don't want to minimize the mystery, either. I think, while some of this may have been exaggerated in the past literature, there are still pictures of somatic pairing in *Drosophila* where the chromosomes are very nicely paired, and there are obvious forces between them over distances of the order of 1 μ or so.

Atwood Some of them are figured as having a region of stretch between one part that is paired and another, for example, in translocations (19) There must be a lot of force involved, because it has stretched the intervening part of the chromosome

Lederberg Is this synaptic pairing?

Atwood Somatic pairing in *Drosophila.* A small gap can be seen between the paired regions.

Stern In any event, there is no question of replication here, because the pairing to which you referred is not between sister chromosomes but between partners of homologous pairs In the Diptera, somatic pairing leads to close, parallel arrangements of homologous chromosomes. At metaphase, they may be separated by considerable distances but when it comes to telophase, somatic pairing becomes very close. What pulls them together in this fashion is the problem.

Levinthal. The kind of chemical forces we've been talking about in connection with DNA chemistry are very short-range forces. The

motion of chromosomes presumably involves long-range forces, which I believe would require either the existence of fibers or some kind of cooperative action involving many electrostatic interactions.

Watson: A final aspect of DNA structure which we might discuss concerns DNA from a small virus called øX174. A related one, also interesting, is called S13 These have been known since the late thirties It was originally claimed, on the basis of rather inadequate data, that the diameter of these viruses was only 150 Å, which would make them very small viruses with molecular weights of about 1 million.

Recently, they have been studied extensively by R. L. Sinsheimer and by I Tessman and E Tessman, and some very interesting facts have emerged First øX174 has a diameter of 300 Å, which places it in the same size category as polio or Bushy Stunt virus It was thought by many of us that, since it was so small, it might be a bacterial virus which contained RNA and not DNA

That there was something peculiar about this virus was first demonstrated by the Tessmans, who grew the virus in the presence of very high amounts of P^{32} and then measured the inactivation of the virus as a result of the decay of the phosphorus (41) They concluded that every decay killed a virus particle, whereas, if the same experiment was done on a virus like T2 or any of the other T-viruses, only one out of ten decays resulted in killing This prompted the Tessmans to make the suggestion that this virus has a single-stranded structure, and I remember considering this suggestion just complete whimsy

But the matter was quite conclusively settled by Sinsheimer (34,35), who grew enough of the virus to isolate it He found that the virus contains thymine, so it is a DNA virus But when he extracted the DNA, it seemed to behave like denatured DNA.

He did base ratios of the DNA and got the following results: adenine, 1 0, thymine, 1 33, guanine, 0 98; and cytosine, 0 75 It is quite apparent that adenine does not equal thymine or guanine equal cytosine These results are beyond experimental error and represent, to my satisfaction, the only noncomplementary DNA. I am referring, of course, to DNA isolated in a gentle fashion There have been other claims of DNA without base pair ratios of 1 0, but I don't think any of them have been convincing

The experiment which I described earlier, of increasing the temperature and measuring the UV absorption, has also been done A 25 per cent increase was observed but with no sharp melting-out point This is the way denatured DNA would behave, or I might say more appropriately, this is the way RNA behaves When the RNA from an

RNA-containing virus, such as polio, is studied, it is found that with heating there is an increase of about 25 per cent in the UV absorption This is a completely reversible phenomenon

The amount of the DNA in the virus is interesting, since the molecular weight of the virus is 6×10^6, and the DNA is approximately one third, or 2×10^6. This is equivalent to about 6000 nucleotides and is the amount of RNA found in most RNA-containing viruses I think, therefore, that this is nucleic acid which is DNA chemically, but which physically behaves as RNA It is probable, on the basis of the melting-out curve, that about 50 per cent of the bases are hydrogen-bonded.

Lederberg. Is that judged from the magnitude of the effect?

Watson: Yes I believe that the øX174 DNA may be our best system for understanding RNA We can't say anything as yet about the replication of this DNA, only that it poses the same problem that arises with the replication of RNA virus, where the base ratios are not complementary However, I would like to have replication by a complementary scheme, such as making a double helix and then, for some reason, doing away with one of the other strands

Lederberg: If you're throwing away a complementary strand, you should keep this in mind in discussing models of mitosis in higher organisms Or did you say that, too?

Smithies. Is the life cycle of øX174 similar to that of other bacterial viruses?

Watson. Yes. It has about a 15-minute latent period and a burst size of several hundred It is a fairly stable virus

Smithies: So that an examination of its over-all behavior would not show that it differed from a more usual kind of virus?

Watson: That is correct It could be a very favorable system for studying the relationship between the DNA and the protein, because it represents the smallest and most homogeneous DNA molecule that anyone is likely to be able to obtain It has a sedimentation constant of 23 S and when it is put in low salt, it breaks down to two components of 14 and 16 S Sinsheimer is unable to explain what this breakdown means

Davis: Is it assumed that this DNA is replicated by the same process of making the complementary strand and then having it, in turn, make a complement? I wonder if the one that infects the bacterium is the parent for all the rest in that burst, and they, in turn, will serve as parents for the next cycle? That would produce an alternation of base ratios.

Watson: If there were an alternation, you might expect to pick up both of them.

Spiegelman: Then, you would get pairing of bases on the average.

Davis You could get synchronization rather than pairing

Watson: We might invent a scheme in which there is preferential combination of one of the complementary DNA chains with virus protein

Spiegelman: And it is always the same one

Watson: Yes What would remain unclear is what to do with the other chain Mutants of the virus have now been obtained, so it should be possible to develop some genetics For a long while, people failed to get genetic recombination, but now the Tessmans (40) have observed some in S13.

Spiegelman. Are they being bothered by a lot of dead phage in crossing experiments?

Levinthal They get a certain amount of recombination Whether the absolute value of this recombination frequency is artificially low because of that, I don't know

Steinberg. Is there a large proportion of dead phage in each transfer?

Watson Yes, more so than with T2 Many particles seem to contain an incomplete amount of nucleic acid, more than half the original In the centrifuge, there are several peaks, of which only the forward-moving peak has high infectivity

Steinberg. T C Sonneborn* has mentioned the large proportion of dead phage and said that it seemed to him that possibly only a very small proportion of the viruses had double helices and were capable of reproduction The large proportion of single-strand phage might be the part which could not reproduce

Lederberg. The original evidence for this being single-strand was the P^{32} *decay kinetics One of the perplexities that hasn't been considered* is the conservation of DNA molecules and of phage DNA aggregates during mitotic replication, on the one hand, and the apparent nonconservation of the entire aggregates in a typical four-strand system of meiosis One would wonder, however, if there is any conservation at all of the input parental DNA in a meiotic system

SUMMARY

Lederberg The previous discussion was a rather comprehensive review of the structure of DNA, and it is obvious we are getting far enough into this to begin to have many very tangible questions, of the

*Personal communication

sort that were not even thought of 5 years ago We are replacing vague ideas about complementariness and replication with rather precise statements, rumored true or false, but at least precise statements, in chemical terms

The most disturbing or provocative comments I have heard at this Conference were the reports of the mechanical fragility of DNA and the effects of manipulation in the laboratory. It becomes perfectly obvious from what was said here that we must treat with great reserve everything that has been said about the size of the DNA molecule I think this year is a great exception There was a quantum jump in the size of the DNA Dr Levinthal was talking about chromosomes as one DNA molecule This is an extremely disturbing statement Could we go back to this question of the large and small pieces of phage, and ask whether we are dealing with exactly the same kind of a situation there or not?

Neel · Could you define what you mean by the large and small pieces?

Levinthal · We found, some years ago, using an autoradiographic technique with nuclear emulsions, that the DNA of the phage T2 consists of one large piece which has a molecular weight of the order of 50 million and a number of smaller pieces of molecular weight of the order of 10 million The method involved the counting of the electron tracks which are produced in clusters These clusters were called "stars" and the star size was a measure of the amount of P^{32} deposited at a point in the emulsion Originally when these results were obtained, we worried about two possibilities of artifact the big piece might be an artificial aggregate of smaller pieces and these pieces might be the result of an artificial degradation of something which was originally larger.

In the last several years, the emphasis has all been on the former sort of difficulty, that it might be an artificial aggregate C A Thomas has been doing a great deal of work to investigate this and I think that he has now ruled out this possibility

In view of Davison's results, the question gets turned around and we must ask whether or not these findings could be the result of an artificial breakdown of a larger unit There is no evidence against this view. However, the evidence does indicate that if the large piece is the result of breakage, it is not a random breakage, but rather the rupturing at the same point in each particle This is shown by the fact (20,21) that when the parental phage was osmotically shocked, the star size dropped to 40 per cent, while the stars among the progeny phage did not change their size when the DNA was released Furthermore, the amount of P^{32} in those progeny phages which did produce

stars was just half of that in the big piece obtained from the parental phage. We interpreted these results as indicating that the big piece divided in half during replication, keeping 50 per cent of its label in one particle while the other 50 per cent went to another The small pieces appeared to segregate among the progeny particles Thus, the results obtained for the big piece were the same as those which were later found by Taylor for the chromosome and by Meselson and Stahl for the DNA molecules of *E coli.*

Lederberg: Your treatments, then, are not drastic enough to break down this material, and the syringe possibly would

Levinthal: The results of Thomas' (42) experiments, which showed that the 40 per cent piece does in fact get broken down by anything which produces velocity gradients, caused some worry about shear degradation

Lederberg I think this long digression is important because it illustrates the rather central role that this technical question plays in the interpretation of all kinds of transfer experiments, etc, as they have been previously carried out

We haven't said very much about the most important aspect of primary structure, the base sequence I think the most provocative single statement that was made about the secondary structure was the remark that Dr Watson made To build an orderly, crystalline structure from the elements which we know are in DNA, it is of prime inportance to keep in mind the fact that the AT pair looks from the outside like the CG pair The double helix can be built around that particular fact That is what is meant by the complementary pairing of two units

There was a good deal of discussion about the extent of disruption of the secondary structure under conditions of denaturation There are still many ambiguities in these questions, such as the little bit of unraveling that many people would like to see happen spontaneously under different conditions I hope, at our next meeting, we can have the results of the formaldehyde experiments that might be able to clinch that particular point, since, so far as I know, that is the first procedure that will measure, not the large fraction that is base-paired, but the very small fraction that is not base-paired

The discussion of replication can be very briefly summarized It fits exactly the scheme that Watson and Crick proposed as a corollary of the structure that they had developed for DNA; namely, that the replication depends on the separation of the two helices and the building of a complementary replica for each of them The cleanest evidence for this at the present time is the work of Meselson and

his associates at the California Institute of Technology. We will now wait for a precise delineation of the sonic experiments, which show that the hybridity of the first generation molecules is really constant throughout the length of each molecule

If it can be shown that this hybridity is maintained, regardless of the size of the piece that is chopped from the DNA molecule with sonic vibration, I think we will have to admit that we are dealing with two strands that are joined together rather than with the competitive models

The only serious difficulty that has ever been brought up for the application of this mechanism of replication to the entire chromosome—and it has been brought up several times—is how a ring chromosome can replicate and how the daughter rings can disjoin from one another. If we are dealing with a helical structure of this sort, I don't think anyone has begun to solve that problem It is one of the stronger incentives for invoking subunits of DNA hooked onto a protein chain, because then we can do what we like with the protein for the separation

On the other hand, if there is a specialized structure such as the centromere, we might put all the burden of the free rotation around this particular point and then, ultimately, we could separate even a wound ring, along the same lines that Dr Levinthal was talking about for the linear chromosome.

Dr Levinthal pointed out that at the present time there are no explicit obstacles to the hypothesis that the entire chromosome in a higher organism has a continuous double-strand helix in its DNA. There are many other materials in the chromosome, and they may play very important functions in the stabilization of the DNA and its biological action, but structurally speaking, there could be a single continuous strand At least the raveling or the unraveling of the pair is not an impossible problem This supposes that the viscosity of the medium through which the long strand must rotate is not much greater than in water, and this may be a reasonable assumption in the circumstances.

It seems to me that again and again we come into very simple problems of organic chemistry and that we don't really know as much about the chemistry of the individual purines and pyrimidines as we would like to for a fuller understanding of the polymers. This problem arose in two or three points. The question of the existence of base pairing between the existent bases in aqueous solution has not been studied. The partial molar volumes are not known. We know very little about the keto-enol transitions of these materials; at least, I think that we would like to know more.

I would like to add just one more point. It might be profitable to

look more diligently for specific chemical reagents for each of the simple bases that are part of DNA I think we are beginning to learn more and more about such problems as mutagenesis by using reagents that are still relatively crude. We have amino reagents in the form of nitrous acid, etc , but at the present time I do not know of any chemical reagent which will attack one of the four bases to the exclusion of the other three I think these would be supremely valuable, not only for analytical purposes but also for attempting specific modifications in the structure of DNA I think these would be important, too, if we are ever going to try to generalize on what kinds of molecules could function in a very similar context, that is to say, we are given, as an existent fact, that there are four bases which occur, and you will see the context of my remark if I say which occur in terrestrial DNA. Is this the limit of all possible choices or could one build other kinds of polymers which would have the same basic properties as the ones we are dealing with here? I think that a detailed understanding of the chemistry of the simple bases is one of the important things that we must possess in order to do this.

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THE GENETIC CONTROL OF PROTEIN STRUCTURE

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AT PRESENT, the abnormal human hemoglobins form the only well-studied system in biochemical genetics where it can be shown that mutations produce chemical alterations in a protein molecule We would like to call this protein molecule the gene product or, at any rate, one of the earliest gene products There is no proof, of course, for the statement that hemoglobin is the primary gene product, but it is certainly one of the very early ones We are in a fortunate position in the case of hemoglobin, because it is the only protein being made in a particular cell, at least, other proteins are made in only very small amounts The protein in reasonably pure form is easily prepared in large quantities, and this has been a very important factor in being able to study the chemistry of the abnormal hemoglobins However, as will be shown later, it can be anticipated that much biochemical knowledge will come from the study of other proteins

We would like to examine the relationship between genes and their protein products and, if possible, to correlate genetic fine structure with the chemical structure of the gene product In the case of hemoglobin, only the fine structure of the product can be examined, but in the microbiological system that Dr Levinthal (67) will be discussing and in the phage system that Brenner (13) is working on, there is an excellent opportunity to study also the genetic fine structure of the system, to subdivide the gene controlling a particular protein, and to determine the fine structure of that gene This is quite impossible in the human hemoglobins for reasons which are only too obvious

The abnormal human hemoglobins are only one example of proteins which occur in different forms as the result of mutations I need mention

only such examples as the tyrosinase of Horowitz (42), where there are five or six different forms of the enzyme which are inherited and which differ in their physical properties, particularly their heat stability. There is also the tryptophan synthetase enzyme of Yanofsky (105), where about a half dozen different forms are known

In another mammalian system, there are the different forms of the β-lactoglobulins (8), A and B, which are found in different strains of cattle in England and presumably other countries There are, of course, different forms of hemoglobin to be found in different animal species, such as mice, goats, sheep, and horses (49). Apart from the horse hemoglobins, these different hemoglobins are inherited in Mendelian manner, but in each case there is only *one* second form known There does not exist a whole spectrum of different proteins as exists in the human hemoglobins. The possibilities for study are therefore rather limited.

In the case of enzymes such as tryptophan synthetase and tyrosinase, there are severe problems of isolation of the protein, and this is a point worth noting We have been extraordinarily fortunate in the case of hemoglobins

First, hemoglobin is a human protein; consequently there is much more interest and many more people are working on it (85) Some of the abnormal human hemoglobins are associated with diseases, and many conditions exist all over the world in which these forms of hemoglobin can be studied In other words, the mutant forms are looked for by a large number of investigators, starting originally with sickle-cell anemia hemoglobin, which will be discussed in more detail later, and which was discovered by Dr Itano in Dr Pauling's laboratory in 1949 (78) Since then, a great many different forms of human hemoglobin have been found (49), but it is because of their medical interest that they have been studied so extensively all over the world.

I have already mentioned the second very fortunate circumstance. It is relatively easy to obtain quantities of pure protein, whereas, for example, Dr. Levinthal has to work hard to get a few hundred milligrams of pure protein That makes a tremendous difference

A third very important factor has been that hemoglobin has been a standard protein for study by protein chemists and biochemists for at least a hundred years (85). There is a vast amount of chemical and biochemical literature on hemoglobin, and while much of the early work is of no direct use at the moment, nevertheless, at the beginning a great deal was known about the chemistry and the structure of the hemoglobin molecule. In these other systems, such as the bacterial

enzymes or the phage proteins, one must deal with a completely new protein and not much is known about its size, its amino acid composition, or the complexity or the number of peptide chains which it has

I would like to discuss abnormal hemoglobins as model substances to be used in this kind of biochemical genetics, and to do so I must review some of the facts of hemoglobin chemistry Human hemoglobins can be divided into three classes the adult hemoglobins, the fetal hemoglobins, and the minor normal hemoglobin components

The normal type of *adult hemoglobin* is hemoglobin A, which corresponds to what might be called the "wild type" hemoglobin. This has many genetically determined variants which are distinguishable· hemoglobins S (the hemoglobin found in sickle-cell anemia), C, D, E, G, and so on

Hemoglobins S, C, and D were discovered by Dr Itano, working with Dr Neel in the case of hemoglobin C (50) The variant hemoglobins are distinguishable primarily because they differ in their electrophoretic mobility, that is to say, they differ in the number of charged groups in the molecule

Hemoglobins A, S, C, and E occur with high frequency in parts of the world Hemoglobin S, of course, occurs mainly in the malaria belt of Africa, with gene frequencies up to 20 per cent in certain regions Hemoglobin C is not quite so frequent, but it also occurs with frequencies up to 10 per cent, principally in Ghana Hemoglobin E occurs particularly frequently in Southeast Asia Most of the others occur rarely (66).

This means that there must be some selective advantage to the hemoglobins S, C, and E in order that they should occur with such high frequency, while there is probably no selective advantage for the other rarer hemoglobins This selective advantage is particularly striking in the case of hemoglobin S, because the homozygous condition results in a fatal disease We have a good example of balanced polymorphism, because there is apparently a natural advantage of the A/S heterozygote with respect to malaria (3,58)

In addition to these adult hemoglobins, there exists the series of *fetal hemoglobins*. A newborn infant has a different type of hemoglobin from that of the adult, it is not known why It is known that the cells containing fetal hemoglobin have a higher oxygen affinity than do the adult cells, but it is not at all clear that this is a property of the fetal hemoglobin itself Of course, to have a high natural affinity for oxygen is a great advantage to the fetus, because at some stage it must transfer oxygen from the maternal circulation to the fetal circula-

tion But what is not clear is that this is an intrinsic property of the hemoglobin. This matter is under active investigation at the moment in various laboratories.

Fetal hemoglobin, of which the ordinary kind is hemoglobin F, is chemically distinguishable from the normal adult series of hemoglobins in a way which will be indicated later. There is an abnormal and inherited variant of the fetal hemoglobin series—hemoglobin Bart's (1)—but it falls into a special category and will be discussed later There have been reports in the literature of a primitive hemoglobin which should come in the developmental series before fetal hemoglobin; that is to say, very early in embryonic life. Halbrecht and Klibanski (35) reported the existence of such a form, and others have also reported similar findings, but this hemoglobin is not generally accepted at the moment

Motulsky. What about the fetal hemoglobins in Southeast Asia and Greece (25,101,102)?

Ingram Those are probably fetal variants.

Fremont-Smith. How long does the fetal hemoglobin last? Does it last after birth?

Ingram Yes, it does If the amount of fetal hemoglobin is plotted at birth, it might be 80 per cent The figure varies from 60 to 80 per cent (101). The rest is adult hemoglobin Then, it falls, and, by the second year, it is down to 1 per cent or less, in normal cases.

The third series of hemoglobins which should be considered is the *minor normal hemoglobin components* which are distinguishable electrophoretically or on a column and which are present in amounts of approximately 2 per cent each. All together, they account for from 10 to perhaps 15 per cent of the total hemoglobin present. There are certainly six or eight of them, and there may be more. These are also under active investigation, mainly by W A Schroeder and R. T. Jones at the California Institute of Technology.

There isn't very much we can say about any of the minor hemoglobins, except one, hemoglobin A_2, of Kunkel (64). This is an electrophoretically distinct minor component of hemoglobin present to the extent of about 2.5 per cent in normal blood. It has been well characterized and will be discussed later.

Levinthal: Is it clear that the number of components is finite, or could it be a continuum?

Ingram I think it is clear that it is finite and not a continuum In other words, there are only a few minor components, but whether there

are seven or ten, no one knows as yet There are not likely to be as many as a hundred

Let me come back now to the *adult* series of hemoglobins, which is the one that is best studied Hemoglobin A is a globular protein, with a molecular weight of 66,000, it is composed of two identical half molecules, each one of which, of course, has a molecular weight of 33,000 This appears directly from the x-ray studies of Perutz (21) at Cambridge and also from certain early sedimentation studies

In the case of hemoglobin A, there are four peptide chains in the molecule, two each of two different kinds, and there are two α- and two β-chains (85) With this sort of molecular weight, there are, all together, about 560 amino acids in the molecule All the evidence so far indicates that these α-chains are of the same length, so that each one contains about 140 amino acids This is somewhat larger than pancreatic ribonuclease, the single chain of which is composed of 124 amino acids It is also larger than egg white lysozyme, but it is still in the range of small proteins

Granted that these four peptide chains are of equal length, how are they distinguished? Schroeder (85) distinguishes them by determining the first two or three amino acids in the chain By definition, the α-chain begins with the amino acid valine, followed by leucine, followed by approximately 138 amino acids The β chain also begins with valine but it is followed by histidine and then by leucine The two α-chains seem to be identical to each other and the two β-chains also seem to be identical

However, while there are certain similarities in the amino acid composition between these two types of chains, there are many differences as well Hill and Craig (36) recently isolated these two chains in pure form by countercurrent distribution and determined their amino acid composition. Some amino acids have similar distribution, but some show significant differences The content of proline in these two chains is identical, and this is of special structural significance

The protein myoglobin of muscle is very closely related to hemoglobin Myoglobin is concerned with oxygen transport and its molecular weight is one-quarter of the molecular weight of hemoglobin, about 17,000 The molecule of myoglobin consists of one peptide chain and one heme group, whereas the molecule of hemoglobin consists of four peptide chains and four heme groups.

If you imagine, in the case of the hemoglobin molecule, that the α-chains are coiled up in some specific manner within the *half* molecule, they are symmetrically related in the two half molecules. This follows

again directly from the x-ray evidence; that is to say, every amino acid which is found in one place has its exact counterpart in the other half of the molecule, as illustrated in Figure 9

Fremont-Smith: Are they mirror images?

Ingram: No, they are not mirror images They are related by an axis of symmetry running at right angles to the diagram and running directly through the molecule, so the halves are related by 180 degrees across the center of the molecule.

The way these peptide chains are arranged in the molecule is highly specific. The alpha helix of Pauling and Corey is a regular way of arranging a polypeptide chain in a fairly rigid spiral or helix, with hydrogen-bonding between the successive turns of the helix, the helix being formed by the peptide backbone of the peptide chain

This kind of straight helix cannot go on indefinitely in a protein molecule such as hemoglobin or, indeed, in myoglobin Every now and again, something happens to disturb this regular arrangement, and the polypeptide chain bends or rounds a corner. One of the points where it becomes impossible to form a regular alpha helix is where the amino acid proline occurs in the polypeptide chain This is the significance of mentioning that both α- and β-chains have the same proline content. Wherever proline occurs, it is not possible to continue to make a regular alpha helix, and it is necessary to turn to some other form of folding of the molecule.

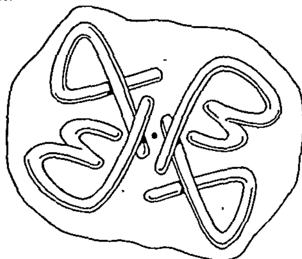


FIGURE 9 Diagram of the human hemoglobin molecules, showing symmetrical arrangement of two α- and two β-polypeptide chains Modified figure, reproduced, by permission, from Ingram, V. M., and Stretton, A. O. W.. Genetic basis of the thalassemia diseases. *Nature, London,* 184, *1903-1909 (1959)*

Hoagland: Does the proline content of myoglobin appear to be the same as a single hemoglobin chain?

Ingram: I think it is similar Figure 10 will give a better idea of what I mean by the coiling up of the peptide chains This is Kendrew's model (61) of the myoglobin molecule at 16 Å resolution It is a model derived from x-ray studies of single crystals The sausage-like objects are not actually the peptide chain itself; they are the peptide chain in a coiled form In certain regions where the sausage is fairly straight, the peptide chain is probably in the form of a Pauling alpha helix Doty (23), by means of optical measurements, showed that myoglobin contains about 85 per cent of its amino acid residues in the form of an alpha helix *

Kendrew has lately refined his structure to a resolution of 2 Å which, of course, is approaching atomic dimensions In some regions which appear to be fairly straight, he can see that the peptide chain is in the form of a right-handed alpha helix, as predicted by Doty There is a hole down the center of the helix, and I understand that Kendrew can see this The points in the molecule where the proline might become important are where the "bends" occur

In Figure 10, the grey projecting object is the disc of the heme group Oxygen attachment presumably occurs near the center of this disc The black knobs in the figure merely indicate the positions in the molecule where Kendrew and his colleagues were able to attach compounds of heavy atoms, such as mercury derivatives This is a device used in the technique of x-ray analysis of this protein, and these knobs have nothing to do with the structure of the protein as such However, the overall impression this picture gives is of a highly specific configuration of a polypeptide chain, arranged in a manner which does not, at the moment, seem to make any sense, but which, nevertheless, is the same in every molecule in the crystal

At this point, I will mention that the four polypeptide chains of hemoglobin are also arranged in a somewhat similar manner This is also some recent work of the other member of the group of x-ray crystallographers in Cambridge, Max Perutz, who has been studying the x-ray structure of hemoglobin for many years He finds, at 5.5 Å resolution, that the hemoglobin molecule looks rather like four myoglobin molecules put together The four subunits only touch one another, they are not intertwined.

This kind of picture does not show that the subunits of the hemoglobin molecule are *exactly* like each myoglobin molecule. We know,

*Personal communication



FIGURE 10 J C Kendrew's three-dimensional model of the myoglobin molecule. The white sausage represents the already coiled polypeptide chain, the grey disk is the heme group The small balls mark the positions of various heavy-atom compounds used in the course of the study (Photograph from Dr J C Kendrew) Reprinted, by permission, from Kendrew, J C, Bodo, G, Dintzis, H M, Parrish, R G, Wyckoff, H, and Phillips, D. C A three-dimensional model of the myoglobin molecule obtained by x-ray analysis *Nature, London*, 181, 662-666 (1958)

from the amino acid composition of the material, that this cannot be so in every detail, but the important thing is that the over-all configuration of the peptide chain is very much the same.

Perutz can also see the four heme groups of hemoglobin which are located on the outside of the molecule. When the four heme groups of hemoglobin react with oxygen, they don't all react equally The first one reacts with difficulty, and the last one with extreme ease This is called heme-heme interaction, and most people have pictured the four heme groups of the molecule rather close together in the interior On this basis, the first oxygen would have to burrow its way into the structure As the structure becomes loosened, the last one finds it very much more open and easy. As it now turns out, these heme groups are *not* close together and they are not near the center of the molecule but on the outside They seem to be as far away from one another as possible, and the whole topic of heme-heme interaction in hemoglobin is wide open again

Given a definite sequence of amino acids in a peptide chain, it appears from what has just been said that the polypeptide chain is arranged in some definite and orderly manner, although it does not make any sense yet We would like to say that the way a polypeptide chain is arranged in space is a direct consequence of the amino acid sequence (the primary structure of the polypeptide chain)

The way in which the peptide chains arrange themselves, as in the alpha helix, is called the secondary structure of a protein, and the way sections of helix arrange themselves in the final molecule is called the tertiary structure of the molecule It is very similar to what Dr Watson was talking about in relation to primary and secondary structure of DNA The tertiary structure is not so evident in DNA, but it is in proteins. It is our postulate that all that genes have to do in the case of, for example, the hemoglobin molecule is to determine the primary covalent structure of the α- and the β-chains The folding up and spatial arrangement follow more or less automatically

Glass. How are the α- and β-chains attached to one another, or are they just loose?

Ingram: They do not seem to be attached to one another by covalent bonds Neither are there any covalent bonds between the different turns of the chain I don't know how they are attached to one another, but I picture it like an antibody-antigen combination They can be dissociated and reassociated, as Dr Itano will tell you They can be pulled apart and put together again under conditions of pH and salt concentration which do not involve the breaking of covalent bonds

Lederberg: How does it happen that they fit together? Is this deter-

mined by the amino acid sequence, which will then permit formation of a tetramer?

Ingram: Yes, this seems likely, because it is a great advantage to the hemoglobin molecule to be a tetramer and to have a sigmoid oxygenation curve with heme-heme interaction. Such a curve enables the molecule to take up the maximum of oxygen in the lungs and to leave the minimum of oxygen on the hemoglobin in the tissues; in other words, this heme-heme interaction does aid the hemoglobin in transporting oxygen Myoglobin, I should say, which has only a single heme group, shows a hyperbolic curve for oxygenation

Lederberg (Tongue in cheek): You don't believe that hemoglobin is an antibody to oxygen, which assumes the configuration instructed by the requirements for oxygen?

Ingram: No, I do not

Atwood. What happens when hemoglobin or myoglobin is denatured? Do you know anything about the way they refold? Both the secondary and tertiary structures must be disturbed when they are denatured

Ingram: Yes, but "denaturation" is a broad term.

Atwood: Let's say, heat it only until some hydrogen bonds break, or enough of them break so that the molecule will assume a different form.

Ingram. I would think that during heating the tertiary structure is disturbed first, because the forces holding the tertiary structure are bound to be weaker than the forces maintaining the alpha helix

Atwood Is this quite reversible, then?

Ingram: At an early stage, it is probably largely reversible. But whether it is 100 per cent reversible, no one knows For instance, the x-ray crystallographers have not looked at reconstituted hemoglobin or myoglobin in any great detail They have reconstituted myoglobin from a fully denatured protein, crystallized it, and examined it by x-ray methods They find that up to a certain limit, the structure must be similar But what the limit is, they don't know

Lederberg: When you say "fully denatured," do you mean the secondary structure is also disturbed?

Ingram: I would say so

Lederberg: That is the best evidence that the primary structure determines the secondary and, in turn, the tertiary?

Atwood: But there is evidence against such an idea, because after denaturation the protein is cooled again and a different structure is obtained in some cases.

Lederberg. Yes, but did you ever get this one specific structure again

or something very close to it? Such findings imply a considerable determinism of that structure by the original configuration.

Ingram: I wouldn't like to say the reformed structure is 100 per cent the same It may be only 95 per cent the same.

Anfinsen: There are situations in which denaturation with urea is quite reversible. Hemoglobin denaturation may involve a sulfhydryl reaction, but the -SH's can still be detected after heating, can't they?

Ingram: Yes, they can, but heat denaturation does seem to be much more drastic Denaturation of hemoglobin by acid, for example, allows the tertiary structure to re-form eventually This would be impossible after prolonged heating Even in completely insoluble and denatured material, with the heme group removed, the protein can renature under the proper conditions of pH and temperature

Levinthal: It seems to me that, in a sense, you overstate the case by assuming it is only the energetics of the final configuration which count There are certainly several other factors which could affect the secondary and tertiary structure However, these still could depend on the primary structure As Dr Anfinsen pointed out, it could be a question of which end started to fold or how the heme group fits in

Ingram. Yes I must say that I think the way in which the heme group fits into the final folded molecule must have a tremendous determining effect on the way the whole molecule folds In this connection, I think you should mention the insulin molecule, which has two polypeptide chains Dr Anfinsen (4) has a very good illustration of this in his recent book There are two polypeptide chains which are linked together by disulfide bonds There is also an internal disulfide bridge within one of the chains Probably helical coiling is prominent within the central region, with a much more flexible region outside The greater part of the molecule is rigidly held together It coiled and then apparently tied up.

Insulin is a very rigid structure as opposed to myoglobin Here, there are no covalent bonds between the turns of the peptide chains This compact and tight model explains at least in part why the insulin molecule is very resistant to denaturation It can be treated brutally and it will not denature easily But myoglobin can be very easily denatured because the folds of the peptide chain are held together only by noncovalent forces

Fremont-Smith: Is there evidence that the heme comes on late, at the last stage of the formation of the molecule, or does the molecule form around it?

Ingram: There is no evidence on this point that I know of, but I think that the polypeptide chain is formed and folds up, all in one con-

tinuous process. I would think the heme group goes on at that same time, and whether or not the heme group goes on or is available to go on may determine the rate at which the chain is produced. In other words, the heme group must be on for the peptide chain to be pulled out of the synthetic machinery

Smithies. But the heme can be taken on and off after the molecule is formed?

Ingram: Under very drastic conditions

Itano. Treatment of hemoglobin with acid-acetone removes heme and leaves a precipitate of acid-denatured globin, which can be redissolved in dilute aqueous HCl solution If this solution is neutralized rapidly, the protein precipitates; however, if the solution is neutralized very slowly or brought to a pH just short of precipitation and allowed to stand for some hours before final neutralization, most of the globin remains in solution It thus appears that the process of folding takes time. Reconstituted hemoglobin results from the addition of a heme solution to solubilized globin The position at which heme attaches is probably the same as that in native hemoglobin, since the reconstituted product crystallizes in the same way as the native protein and combines reversibly with oxygen

Fremont-Smith. And it is identical with the original?

Itano. It is hard to prove identity

Fremont-Smith: There is no recognizable difference from the original?

Ingram. I think there are slight differences Whether these are differences due to technique or inherent in the system, no one knows

Smithies: Would you say that the differences in the crystalline structure of the "renatured" protein were a result of the presence in the crystal of a small number of molecules which are badly wrong, with the rest normal, or that all are slightly wrong? Is it possible to say?

Ingram I cannot answer that I am not sure that any x-ray crystallographer could tell

Lederberg: This particular topic is of prime importance. It has everything to do with the kind of information that must be coded into the genes. Many of us would like to feel that it is sufficient to relate a gene to an amino sequence, and that may be coloring our thinking about the chemistry of the proteins Trying to figure out how the nucleic acid could determine the tertiary structure in any direct way is an almost impossible problem.

Davis: I suppose we would all feel more confident that the primary structure determined the secondary and tertiary structures if 100 per cent

renaturation was obtained But this may be too much to ask After all, a protein that is forming its secondary and tertiary structures while being unpeeled from the template is undoubtedly facing a quite different problem from a completed chain that has been unwound and tangled up by denaturation and now has to find its way back Perhaps the remarkable thing is that any degree of renaturation at all is obtained, rather than that complete renaturation is not obtained

Anfinsen Do you think that is the case in reducing and reoxidizing ribonuclease?

Ingram Why don't you go into that now?

Anfinsen: In the case of ribonuclease, the molecule consists of a single chain, as shown in Figure 11 It is held together by four disulfide bridges and it has 124 amino acid residues (6,38,39) Since the structure is well known, we put it through a number of chemical procedures without worrying too much about side reactions The disulfide bridges can be reduced with sulfhydryl reagents and urea, so that a fully extended

FIGURE 11 The structure of bovine pancreatic ribonuclease (6, 38, 39), and some examples of relationships between its structure and function Reprinted, by permission, from Anfinsen, C B, and White, F H Jr *The Enzymes*, 2nd Ed, Vol V P. D Boyer, H A Lardy, and K Myrbäck, Editors New York, Academic Press (In press)

chain is obtained with 8 -SH groups along the chain. These can be stabilized by reaction with iodoacetic acid, to give the S-carboxymethyl derivatives Then, the total information in the chain is stretched out in a linear fashion (with modified sulfhydryl groups).

For chemical work, we have stabilized -SH groups by covering them. However, for studying the problem of reversible folding, the reduced chain is isolated under conditions that prevent any chance reoxidation and then resuspended or redissolved in phosphate buffer around pH 8. This solution is gently bubbled with oxygen

These experiments have been done mainly by F H. White (88,103), who has found that he can regenerate, with about 80 per cent yields, material which is fully active and which behaves on columns like the original starting material In some experiments, White has actually gotten as high as 100 per cent recovery and activity. The only reservation that must be made—and this relates also to the hemoglobin regeneration —is that the physical properties of the refolded ribonuclease are perhaps slightly different The reoxidation product seems to be slightly more globular and perhaps slightly more helically coiled, although this isn't certain But the activity is completely normal *

It is possible specifically to cleave off the first 20 residues of the ribonuclease chain with subtilisin This has been done by Richards at Yale University (81) There is then a fragment of 20 plus a fragment of 104, "the core," all of the disulfide bridges being in the large piece. The two parts are enzymatically inactive until mixed, but complete regeneration of enzyme activity results from a 1 to 1 mixture Dr. Haber and I have observed† that the large "core" will also undergo the reversible reduction-reoxidation reaction.

Smithies. As far as the final structure is concerned, have you studied the antigenicities of the whole ribonuclease?

Anfinsen: R. K Brown and F. H White** have tested the immunological characteristics of reoxidized and original protein and find them to be the same

Spiegelman: Has anything been done to establish the nature of the complex between the two pieces? They must associate in a specific way.

Anfinsen. Various studies have been made on modifying the tail. Dr. Brown knows, for example, that the amino groups are unimportant except for the terminal alpha

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*EDITOR'S NOTE Dr Anfinsen would like to add the following to his remarks at the Conference:

More recent unpublished studies by Dr White on regenerated ribonuclease make it very likely that this material is the same as the original native protein

†Unpublished data

**Unpublished data

Spiegelman. Have sedimentation studies been done with the tail put back?

Anfinsen: Yes, that sort of thing has been done The regenerated material is superficially the same as the original

Glass: Does it re-form the peptide linkage?

Anfinsen: No, that remains ruptured

Glass They are active when separated?

Anfinsen The enzymatic properties and most of the physical properties are identical

Spiegelman. Therefore, they must complex in some way

Davis: Then, I gather that the tertiary forces are great enough in this case to ensure realignment

Anfinsen: There are two different subjects being discussed One has to do with the information necessary for disulfide bridging These experiments of ours suggest that the coded information in the form of amino acid sequence is enough to determine at least the major share of whatever ancillary structure this protein has The other question has to do with the fit between this tail and the core

Spiegelman: Is it possible to fold that in any other way and use up all the cysteine?

Anfinsen About 105 combinations of half-cystine residues are possible Presumably a vast number of three-dimensional coiled forms might satisfy the pairing of 8 -SH groups to form 4 -SS- bonds (100)

Spiegelman And match the cystine measurements? I place that restriction on it.

Anfinsen. Figure 11 is in two dimensions and doesn't permit three-dimensional guessing In two dimensions, this is the only way to draw in disulfide bonds without having the chains overlap and cross one another

Ingram Having found one proper one, the rest will follow more and more easily

Anfinsen: It appears, incidentally, from White's studies, that lysozyme may do the same refolding trick

Lederberg Have you tried making structures with pleating?

Anfinsen. A model can be constructed from what is known about this enzyme The tail is arranged, in a model that Scheraga has constructed (83), in such a way that it crosses over four other chains and makes about five points of contact, two of which involve tyrosine carboxylate hydrogen-bonding There are also a number of scattered electrostatic bonds

Lederberg: Can an artificial tail which has the minimum requisite properties be synthesized?

Anfinsen: This has not been done but it seems feasible.

Marks. Would it not be a consequence of this reasoning that differences in properties of a mutant protein from that of a wild type must reflect differences in amino acid sequence and not merely changes in secondary or tertiary structure of the protein?

Anfinsen: That would be the inference that I would draw.

Lederberg. It is not obvious how you could get any change in tertiary structure without changes in primary structure.

Anfinsen. I would guess that it *would* have to be in primary structure However, preliminary studies on species difference in ribonuclease indicate that some rather large changes can be made from species to species, and these make no difference in catalytic activity or in secondary or tertiary structure For example, lysine can be substituted by glutamic acid (*i.e*, beef vs sheep ribonuclease) at one point without any obvious physical or catalytic change (5,7)

Lederberg: I should like to say a word about antibodies, because this kind of discussion gives us a setup in which we can make some sensible hypotheses about the nature of antibody specificities and how they are formed

It has been a traditional picture, or at least one that Linus Pauling and others of great insight have cultivated, that the antigen comes into the organism and somehow instructs the folding of a predetermined amino acid sequence to make a complementary molecule. I found this philosophically unacceptable for a long time, and no one has ever succeeded in reproducing the conditions of the original experiment (77), which indicated the production of specific antibodies by the denaturation and renaturation of γ-globulin in the presence of an antigen *in vitro*.

It is rather peculiar that such a wide variety of substances is able to convey this type of instruction It is very much in contradiction to the present discussion, in which we speak of the primacy of primary structure as the basis for the three-dimensional shape of a protein, and no one would reasonably impute to miscellaneous antigens the capability of altering the primary sequence of amino acids of the corresponding globulin.

If, in fact, the burden of the specificity of the different antibodies is in sequence, given that that sequence determines tertiary structure, how is this very large variety of information obtained? One way to do this is to suppose that there are 10,000 or 20,000 genes for globulins

arranged in some way, all of them potentially capable of working in every antibody-forming cell The antigen would then come up to one of them and say, "Go ahead and work" This seems rather bizarre

This line of reasoning leads inevitably to a selective hypothesis of antibody formation Different cells would have different genes for antibody formation, each potential antibody-forming cell would make a particular kind of antibody globulin, and the function of the antigen would be to select not one gene among many but one cell among the total cell population, to stimulate it to multiply and to produce a lot of protein (14,65)

Anfinsen· "Inevitably" is a strong word I was thinking, for example, of the sort of theory that Karush has suggested, which I think is not too bad. He says that there are enough -SH groups in globulin so that the number of possible combinations of half-cystines to form -SS-bridges is really quite astronomical The single primary structure can lead to, or would permit, an almost infinite number of foldings, depending on what does the folding

Lederberg: This is another case in which it is possible to obtain considerable insight into a genetic phenomenon by examining the chemistry of antibody globulin, because if it can be conclusively demonstrated that there is no sequential difference among antibody globulins, there is no very good reason to think that the specificity is genetically determined

On the other hand, if the antibody globulins are sequentially different, we would almost have to believe that the specificity is genetically determined Karush (59) has done the very interesting experiment of denaturing antibodies with high concentrations of urea, and the rotatory changes show considerable change in structure. Yet, these snap back into place When he uses disulfide-reducing reagents as well, he loses all the antibody activity I don't think he was looking for the possibility of denaturing under these conditions, and I think that experiment really should be done very carefully and exhaustively Under these conditions, it should work

Davis: While the case that Dr Lederberg makes for a genetic determination of the structure of each antibody is a plausible one, I would like to put in a word against placing too much confidence in the assumption that the same mechanism of determining secondary and tertiary structure can be extrapolated from enzymes or hemoglobin to all proteins It is evidently in the interest of the cell that enzymes should have as fixed and inflexible a structure as possible, and it is gratifying to begin to find reasons to believe that the gene-determined

primary structure uniquely determines the secondary and tertiary structure of these proteins However, if the cell should find it advantageous to make certain proteins with a more flexible structure, it is not hard to conceive that there might be some polypeptide chains that could fold equally easily into any of a variety of secondary and tertiary configurations The more classical hypothesis of antibody formation by alternative folding would require such proteins; and while the hypothesis may or may not be correct, it should not be discarded on the basis of evidence from other proteins that have been selected for precisely the opposite property

Ingram. I should now like to consider some of the chemical techniques, directly derived from the work of Sanger, which have been used to study the structure of human hemoglobins such as hemoglobins A and S. From Dr Neel's work (73), we knew that the difference between hemoglobins A and S is an inherited one. From Dr Itano's work (78) on the electrophoretic behavior of the hemoglobins, we knew that it is a chemical difference This is not immediately obvious, it might have been a difference in folding only. Originally, that was considered to be a very distinct possibility The chemical difference which Dr Itano found is in electrophoretic mobility of the two hemoglobins, which means a difference in the number of available positive and negative charges on the surface of the protein molecules

Smithies. Or a difference in size

Ingram: I don't think we should say that, because it was already known at that time that the size of the altered hemoglobin molecule is the same

Smithies Yes, in this case, but as a general point, the difference in mobility may be caused by either Care should be exercised concerning this, because there may be cases where differences in electrophoretic mobility can be distinguished, where there is no difference in total amount of material and charge but only in organization or folding

Ingram: Yes. The difference in folding, and the difference in molecular size, both leading to a difference in the number of available charges, are very closely related and quite distinct from the other phenomena, where a difference in primary structure leads to a difference in electric charge.

Smithies· The point I wish to make is that two molecules differing in their shapes may differ in their mobilities even though they carry identical charges and have identical masses

Ingram· The other outstanding characteristic of hemoglobin S is, of course, the one from which it derives its name, the formation of sickle

cells. Sickle cells are formed because reduced hemoglobin S has a very much lower solubility If the oxygen is removed from cells containing hemoglobin S, the hemoglobin S precipitates in paracrystalline aggregates, and the cells become distorted as a result and assume the characteristic sickle-cell shapes Such cells are easily broken and destroyed by the spleen, hence the anemia The clinical picture of sickle-cell anemia can be laid in very large measure to the reduced solubility of the deoxygenated form of hemoglobin S As Pauling put it, this is a "molecular disease," a disease at the molecular level However, I don't know whether or not all clinicians agree that all the symptoms of the disease are caused by the reduced solubility of this form of hemoglobin

Neel: There is evidence that more than abnormal hemoglobin alone is involved in sickle-cell anemia From the reports, some subjects appear to have only hemoglobin S but maintain hemoglobin levels at about 11 gm per cent, of which 5 to 20 per cent is fetal hemoglobin, they do not appear to suffer from the usual ill effects that are associated with having only hemoglobin S (24,75) This may be tied in with the oxygen dissociation curve, which is usually, but not always, abnormal in the erythrocytes of sickle-cell anemia We don't know why there should be this difference

Ingram· Presumably the final cell population is a result of many factors of which hemoglobin formation is only one The rate at which it is produced, the rate at which cells are produced, and the rest of the environment must come into the final clinical picture But isn't it correct to say that, wherever there is sickle-cell hemoglobin, a great deal of the clinical picture can be laid to this?

Neel: Yes, but perhaps not *all* the clinical picture There are subjects whose hemoglobin is apparently largely or entirely type S who are doing surprisingly well We don't know why

Fremont-Smith Are there any other abnormalities?

Atwood: There is certainly a difference in kidney function

Davis Is it immediately obvious that hemoglobin S causes this difference in tubular function?

Itano· Although it has been shown that hyposthenuria is associated with sickle-cell trait in the absence of other symptoms, the role of hemoglobin S is not known (60) On the other hand, the severe manifestations of sickle-cell disease can be traced back to the abnormal tendency of deoxygenated hemoglobin S to aggregate.

Neel: The hyposthenuria story might be a secondary manifestation.

Glass: But is there any assurance that the high hemoglobin levels in these individuals are due to the same genetic locus?

Neel. In point of fact, we think that the high hemoglobin levels of many of these individuals are the result of the interaction of one S gene with another gene, perhaps the high fetal gene, or the high A_2 gene. Whatever the genetic basis, the great majority of their hemoglobin is type S, but yet for some reason they don't show the usual consequences we associate with this state This might imply that there are other effects of the sickle-cell gene with which we haven't come to grips

Smithies I disagree with one of the steps in your argument, Dr Neel. I don't know whether or not you really intended it when you said that the better position of some of the subjects who are homozygous may be a result of other actions of the sickle-cell gene Don't you think it is more likely, as Dr. Glass was saying, that they are different in some other part of their genetic make-up?

Neel· We ourselves have used that argument in the past, but an individual may have all hemoglobin S and yet do very well, so that something more than hemoglobin S may be involved in the clinical syndrome that we call sickle-cell anemia

Wagner Has much work been done in determining the oxygen dissociation curves of S and A under different conditions of pH, temperature, etc ?

Ingram· Nothing really very trustworthy.

Wagner· It is germane to the rest of this discussion because, if the rest of the genotype were affected, quite a different effect of S might be expected in one background, because of a change, let's say, in oxygen dissociation, as compared to another background.

Neel. Don Rucknagel,* now with us at the University of Michigan, is working on this quite extensively He finds a difference in the oxygen dissociation curve depending on whether one works with the intact cell or with a suspension of hemoglobin Precisely what this means no one is quite prepared to say as yet.

Itano The oxygen equilibrium of normal adult and fetal human hemoglobin also depends upon the method of preparation Different results have been reported with whole blood, washed cells, undialyzed hemolysates, and dialyzed hemolysates (2)

Fremont-Smith· Do I understand there is much more fetal hemoglobin in these cases than you would otherwise get in an adult?

Motulsky There is a special group of cases where adults have a high amount of fetal hemoglobin

Neel· Some adults with this disease may have as much as 20 per cent or even higher fetal hemoglobin.

*Unpublished data

Atwood: If there are genetic modifiers that alter the severity of the disease, one would expect to find them in places where the incidence of S hemoglobin is very high. One would expect to find the severe cases in recently mixed populations, where the S gene would have gotten away from its modifiers Is that the case, Dr Neel?

Neel: I don't think we know My impression is that the course of sickle-cell anemia in African natives is much the same as in American Negroes In this country, I found no correlation between the apparent amount of Caucasian admixture in Negroes and the severity of sickle-cell anemia (74) This, of course, gets into the question of what happens to balanced polymorphic systems in the presence of hybridization

Lederberg Is there any conflict here? There is nothing of clinical consequence that flows from any source other than the change in hemoglobin?

Ingram One of the primary effects of the possession of sickle-cell hemoglobin is that there is a tremendous change in the rate of manufacture of hemoglobin and erythrocytes, and this is an effect which is not reflected in quoting percentages of hemoglobin in the blood Yet, it may affect the clinical picture enormously

To return to the chemical studies, we knew that the electrophoretic difference is almost certainly a very small difference between these two molecules, because amino acid analyses of the two proteins were indistinguishable, as were x-ray studies of the two hemoglobins and end-group analyses of the peptide chains

We have investigated these hemoglobins by the fingerprinting technique, which is a method devised to characterize in detail the chemical structure of a protein (47) Sanger (82) has shown that wherever the amino acids lysine or arginine occur in the polypeptide chain, attack by the proteolytic enzyme trypsin will split the chain at just that point The peptide bond on the carboxyl group of lysine or arginine is attacked by trypsin and, as far as we know, no other bonds at all are attacked

In the whole molecule of 66,000 molecular weight, there are two identical subunits of 33,000 Each one contains some 26 arginine and lysine residues Attack with trypsin gives a mixture of 28 different peptides, which are then characterized by a two-dimensional combination of paper chromatography and paper electrophoresis These two-dimensional chromatograms prepared from hemoglobin S and from hemoglobin A can be readily compared Figure 12 shows the fingerprints of hemoglobins S and A The individual peptide fragments occupy identical positions on the two chromatograms, with the exception of one. Peptide No. 4 in hemoglobin S has a new position as compared to hemoglobin A

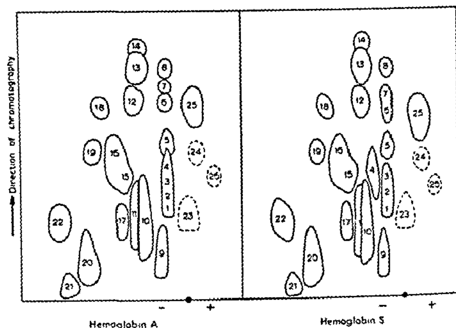


FIGURE 12 Fingerprints of trypsin digest of hemoglobins A and S Tracings of a pair of fingerprints showing the system for numbering peptide pairs Dotted lines indicate peptides which only become visible after heating the chromatogram Reprinted, by permission, from Ingram, V M Abnormal human haemoglobins I The comparison of normal human and sickle-cell haemoglobins by "fingerprinting" *Biochim et biophys acta* 28, 539-545 (1958).

More detailed examinations of the individual peptides include a comparison of their amino acid composition and such factors as staining properties with specific reagents Only this one peptide difference between hemoglobin S and hemoglobin A has been detected so far

Figure 13 shows a similar sort of pattern for hemoglobins A and C and other hemoglobins Peptide No 4 is in its usual position in hemoglobin A, but it is absent in C However, there are two new peptide spots Again, in hemoglobin C, peptide No 4 has a different position, or is absent, and this sort of evidence points to peptide No 4 as being the only difference between normal hemoglobins A and C (45) The only effect of the gene mutations on the primary structure of these proteins would seem to involve this single peptide

Figure 13 also shows a comparison between hemoglobins A and E (45,46) Peptide No 4 is present and unchanged in hemoglobin E, but peptide No 26 has disappeared. This peptide has a weak ninhydrin

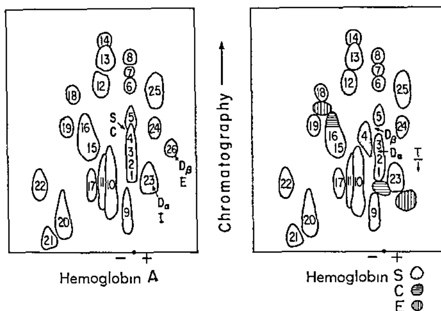


FIGURE 13 Summary tracing of fingerprints of tryptic digests prepared from hemoglobins A, S, C, D_α, D_β, E, and I The peptides which carry mutational alteration are indicated by shading

reaction although it is easily recognizable by its content of arginine Its place is taken by two peptides The suggestion at this point is that in hemoglobin E, there also seems to be only one peptide change in the molecule

In Figure 14, the trypsin digests of hemoglobin A are compared with hemoglobin D Hemoglobin D is rare and it occurs in widely scattered areas throughout the world It behaves like hemoglobin S electrophoretically, but it does not have the reduced solubility Most cases of hemoglobin D that have been described do not produce any very severe disease, and in some cases none at all, nor does hemoglobin D have any detectable selective advantage The numbers are not very large

We examined three samples of hemoglobin D supplied by Herman Lehmann in London from three different subjects, a Gujarati Indian, a Sikh Indian, and a Turkish Cypriot These were all characterized as hemoglobin D electrophoretically, but they all turned out to be different on this kind of examination or on the two-dimensional fingerprints

Figure 14 shows a comparison, side by side, of a normal hemoglobin digest with our first hemoglobin D digest It was immediately obvious that peptide No 23 was missing But in the next hemoglobin D to be

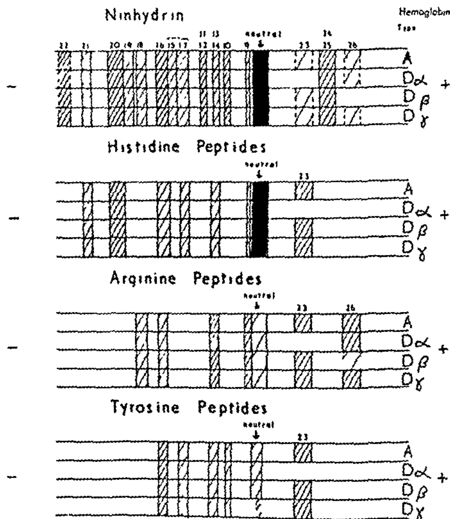


FIGURE 14. Comparison by one-dimensional paper electrophoresis at pH 6·4 of tryptic digests of hemoglobins A, D_α, D_β, and D_γ, with specific color tests for certain amino acids. Reprinted, by permission, from Benzer, S., Ingram, V. M., and Lehmann, H. Three varieties of human haemoglobin D. *Nature* 182, 852 *(1958)*.

examined peptide No. 23 was present in its usual position, and peptide No. 26, which disappeared in hemoglobin E, has also disappeared here. In the third hemoglobin D sample, both these peptides were present Therefore, it was quite clear that these three samples of hemoglobin D from three different populations are different mutations resulting in different abnormal hemoglobins.

This will probably be a constantly recurring feature of all work on inherited abnormalities in proteins. As our methods become more discriminatory, the different mutants which were thought to be the same by previous criteria may turn out to be different If there is no selective advantage in a hemoglobin D mutation, it presumably will not become very frequent. Among these rare mutations, mutant proteins are apt to be found, they look the same at first, but on closer analysis do not turn out to be so

This brings me to the point that Dr Neel is constantly raising, that is, are our hemoglobin A samples taken from different parts of the world really the same? They all seem to be normal hemoglobin, and they are all electrophoretically the same So were these three samples of hemoglobin D

To study this question of the hidden variability of protein structure and the hidden mutations, we, at Massachusetts Institute of Technology, have just begun a survey of the apparently normal hemoglobins We want to examine hemoglobin A samples from all over the world by fingerprinting and similar methods to see just what the hidden variability in an apparently normal protein might be

Neel· There is a genetic point that might be made here, Dr Ingram You said that as far as is known at the moment, hemoglobin D is essentially a neutral trait, it is not being selected for as are hemoglobins S and C, and it is not increasing However, given a finite mutation rate and a history of man on earth of a million years, mutation pressure alone might be expected to build some of these variants up to a considerable frequency There is implied, I believe, a question as to why these variants are not more frequent if they are completely neutral

Ingram· What frequency would you expect?

Neel Who can say? But that is a technical point

Ingram Yes I think this point can be taken a little further with the hemoglobin D. It is possible to make a chemical argument whereby it can be said that any mutation in the protein molecule which leads to reduction of a unit charge of 1 would appear on electrophoresis as hemoglobin D In other words, in a random distribution of hemoglobin variants, this class of hemoglobin D can be expected to be among the most frequent Therefore, if we look for a completely random distribution of hemoglobin abnormality, this class will come to the foreground

Although the hemoglobins D are rare, they are not as rare as some of the other rare hemoglobins, so that, perhaps, the building-up process which you suggested has operated in some cases

Atwood: Wouldn't you expect neutral substitutions to be more frequent than a change of one charge?

Ingram. Yes, I would from a purely statistical approach, but on the other hand we are not quite in a position to say anything about them

Davis What is the frequency of these various hemoglobins D?

Ingram. I have done only these three. Lehmann (66) has calculated that there are about a million people in India alone who are affected by hemoglobin D, but at that time it was assumed to be just one substance. There are a fair number of people in India who are affected by some form or other of hemoglobin D

Itano Cases of apparent homozygosity in the gene for hemoglobin D have been reported Simultaneous presence of hemoglobins S and D is also associated with anemia In fact, the first known occurrences of hemoglobin D were observed in a family in which two of the children had sickle-cell disease associated with S and D (97)

Ingram: Would you say that the anemia associated with some of these D's is mild?

Itano Yes

Stern Are the individuals who are homozygous D known to be homozygous for the same D?

Itano That is not known

Ingram: Figure 15 shows the structure of peptide No 4 from hemoglobins A, S, and C This is slightly different from the one originally published This structure is interesting because it begins with valine, histidine, and leucine The β-chain of hemoglobin also begins with

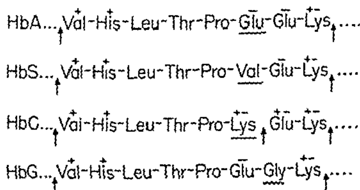


FIGURE 15 Structure of the No 4 peptides from hemoglobins A, S, C, and G (Val = valine, His = histidine, Leu = leucine, Thr = threonine, Pro = Proline, Glu = glutamic acid, Gly = glycine, Lys = lysine.) Hemoglobin G based on Hill, R. L, and Schwartz, H C A chemical abnormality in hemoglobin G *Nature, London* 184, *641-642 (1959)*

valine, histidine, and leucine. In addition, it has recently been shown conclusively that this peptide No. 4 is the beginning of the β-chain.*

Figure 15 shows that amino acid No. 6, a glutamic acid, has changed to valine in hemoglobin S and to lysine in hemoglobin C. The rest of the peptide molecule is the same. It is evident from this why, in hemoglobin C, there are two new peptides appearing, because the introduction of a new lysine residue offers another point of attack to the trypsin enzyme. In hemoglobin E, a similar situation occurs, leading to two new peptides again.

This exchange of glutamic acid for valine is, as far as we know, the only effect of the mutation, and it is one out of 280 amino acids which can be exchanged. It illustrates the very fine control of the gene over the final protein structure. So does the change from glutamic acid to lysine. It is a single amino acid substitution in the whole molecule, as far as we know.

I should like to point out that there is genetic evidence to indicate that the hemoglobin S and C mutations are allelic or very closely linked. In view of the fact that they affect the same amino acid, they could hardly be more allelic.

The bottom line of Figure 15 shows hemoglobin G, which is derived from a family studied originally by Neel, Schwartz, and others (86). It is a critical family in genetic analysis, and I think we might discuss the genetics a little later. Let me say, however, that it is a family in which we find two abnormal hemoglobins, G and S. I want to concern myself only with the chemistry of these at the moment, which has been done by R. L. Hill (37). The original genetic evidence, based on this same family, indicated that the hemoglobin G mutation was nonallelic to the S mutation. Yet, to his great surprise, Hill found that there had been a glutamic acid to glycine change in the very next glutamic acid of peptide No. 4 (Figure 15). At the moment, we are in the apparently paradoxical situation—at least it is paradoxical to those of us who believe in the "dogma"—that two seemingly nonallelic mutations can result in amino acid substitutions in two neighboring amino acids.

Watson: What is the evidence that they are nonallelic?

Neel: This is evidence based upon a single child. Unless we have run afoul of either technical error or nonpaternity, the interpretation we gave that family is one we must stand by (86). Essentially, it involves an individual who has both hemoglobins G and S but who failed to transmit either of these characteristics to a child. This would imply nonallelism.

*Schroeder, W. A. Personal communication.

Atwood. Wouldn't you expect neutral substitutions to be more frequent than a change of one charge?

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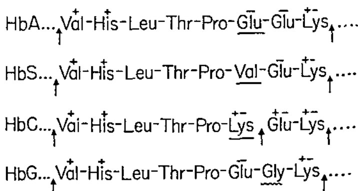


FIGURE 15 Structure of the No 4 peptides from hemoglobins A, S, C, and G (Val = valine, His = histidine, Leu = leucine, Thr = threonine, Pro = Proline, Glu = glutamic acid, Gly = glycine, Lys = lysine) Hemoglobin G based on Hill, R L, and Schwartz, H C A chemical abnormality in hemoglobin G *Nature, London* **184**, *641-642 (1959)*

The pedigree of this family is shown in Figure 16 It is easiest to start with the little girl, III-5, who has the picture of heterozygous thalassemia Her father is normal, and her mother, II-7, has a cytological picture which appears to be that of thalassemia Electrophoretically, she has either all hemoglobin G or a hemoglobin so similar to hemoglobin G that there is no better term for it at the present time

This woman has a sister, II-5, with the identical picture of thalassemia and hemoglobin G It is of interest that, although we could not study the mother of these two women, we could study the father, I-3, who is normal. Therefore, both the thalassemia and the hemoglobin G were transmitted from the mother This would be evidence for nonallelism of these two factors An interesting point is that II-5 and II-7 appear to have all hemoglobin G, even though they are only heterozygous for the gene This gets us to the question of thalassemia interactions with the hemoglobin G locus

This woman, II-5, whom we believe to have the genes for thalassemia and hemoglobin G, married a man with sickle-cell trait, three of whose siblings also have the trait They have two children One of them, III-3, has the sickle-cell trait and by electrophoresis has hemoglobins G and S It is noteworthy that he shows nothing more severe than the clinical picture of the sickle-cell trait In other words, G functions as normal hemoglobin in this particular respect This man transmitted the hemoglobin G to his daughter

His brother, III-1, has the clinical picture of sickle-cell anemia This is one of two critical points in the interpretation This man appears to have hemoglobins G and S He married a normal woman and his child, IV-1, has thalassemia but neither hemoglobin G nor S The failure to transmit either hemoglobin G or S is evidence for nonallelism of the two genes concerned *

Lederberg What about other genetic markers?

Neel. We did extensive blood typing and have no paternity exclusion This family is being very carefully restudied at this point by H Schwartz and R Hill of the University of Utah Medical School because it is at the moment such a very critical family to our whole thinking about these matters

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*EDITOR S NOTE Dr Neel would like to add the following to his remarks at the Conference

Hill has very recently reported that he has been unable to isolate the 'hemoglobin G polypeptide from the individual s hemoglobin after trypsin digestion, and he has suggested that the fraction which migrates as G' may be a collection of minor components in increased proportions *Proc Symp on Methodology in Human Genetics, 1960 (In press)*

Ingram: The chemical finding (37) is not derived from the critical member of the family.

Watson. Are you sure that the one amino acid exchange is the only difference in hemoglobin G, or could this involve some hidden mutations?

Ingram: I don't know how far Hill has taken this work; it is the only one he has found Nor do I know how critically he has examined all the other peptides

Motulsky. If you believe the "dogma," there should be no more changes

Lederberg: There is no reason why the genes, as found in natural populations now, might not have been subjected to more than one mutational event in sequence I think that is something you just have to look for.

Glass. Isn't the probability of recombination between the adjacent sites going to be very much lower than the probability of exclusion of paternity?

Neel. I would go one step further I believe, in this particular instance, that the possibility of recombination between adjacent sites can almost be disregarded.

Lederberg: If you say this on the basis of one test, then you've got no test of allelism in man.

Wagner: Have peptide fingerprints been run on these individuals?

Neel: The fingerprints have so far been run only on the one woman, II-5, who has the thalassemia gene, too, and who, electrophoretically, appears to have all hemoglobin G.

Wagner: You should run one on the child, IV-1, to be sure

Neel I think Hill and Schwartz hope to work through this entire family by fingerprinting.

Wagner: But you must be very sure of that child

Ingram. Yes, but both hemoglobins G and S are electrophoretically quite easily distinguishable, not from one another but from hemoglobin A, and that child shows neither The critical person is the man, III-1. If this man really does have both the hemoglobin G which Hill described and the S, and his child has neither, the nonallelism argument must stand.

Stern: Isn't the argument for true paternity strengthened by the fact of the thalassemia of the child?

Neel: Yes, that's right

Stern: What is the probability of thalassemia in case of nonpaternity? Are they Negroes?

Neel: No, this is a family of Sicilian extraction And if this was a case of nonpaternity, there would be a chance of 1 in 25 that the outsider might leave a thalassemia gene, assuming he is also likely to be Sicilian

Cotterman: Might it also be valuable, while fingerprinting the members of this family, to have some *real* fingerprints taken? I was never so convinced that C. A Cotterman was my own biological parent until I saw a complete set of his dermatoglyphs, and compared some very unusual patterns of his soles and thumbs with those of my own I think where one is trying to convince oneself of paternity or maternity, or parenthood in general, there is nothing better than dermatoglyphs for that purpose.

Lederberg. I wonder if the critical member of the family mentioned by Dr Ingram has a gonadal component which is AG rather than SG, for example, which his own parentage would allow These things do happen. It's a little less remarkable if it happens in the family that you're interested in that there are recombinations between adjacent sites, but he may have gonadal tissue which has the constitution that you infer from his progeny, without necessarily having the same somatic phenotype That is a possibility we have to take into account very much

This would reduce the possibility of exclusion of paternity, because it means the genes for which he is segregating are not the random ones of the population but the restricted ones from his own parents, so the exclusions perhaps ought to be looked at from that point of view If they have not been excluded, you can't say anything here

Another possibility occurs to me I think that child's hemoglobin should be fingerprinted, because it is just conceivable that without thalassemia, hemoglobins G and S are somehow complementary, to give a normal-looking electrophoretic pattern

Neel: I think we can rule that out with one of the other members of the family, the GS male, who shows about 50 per cent hemoglobin G and 50 per cent S We don't think he has thalassemia I know how weak a series of one is, but this is the nature of human genetics, and to find a 3 cross point for man is not easy

Atwood: Are you absolutely sure that III-1 has no hemoglobin A?

Neel. This raises the question of very minor components Of course, we were wondering about the obvious fact that if there were two loci, why wasn t there some hemoglobin A coming through, as there is in the case of the Ho-2 (Hopkins-2), which will be discussed later? We can only say that on the face of our data there was no hemoglobin A

α-hemoglobin gene, making α-peptide chains, beginning with valine and leucine, and the β-hemoglobin gene making the β-chain, beginning with valine, histidine, and leucine.

Peptide No 26 is known to be located somewhere in the β-polypeptide chain We don't quite know where. But it carries both the hemoglobin E and D_β mutations. We don't know as yet which amino acid is altered on the D_β mutation Purely schematically, we would say then that at the corresponding point in the hemoglobin gene, there is a mutation for hemoglobin E and also for D_β, either on top of it or right next to it The peptide No 23 is in the α-chain of hemoglobin, although we don't know exactly where This is the same peptide which disappears in hemoglobin D_α and in hemoglobin I, which I havent mentioned yet.

There is a great deal of work involved in locating these peptide changes in the α- and β-chains, and I don't think we should go into it in chemical detail at this time. It will be discussed later by Dr. Itano when he talks about dissociation and reassociation of hemoglobin molecules He has a very sensitive method for telling whether a particular hemoglobin abnormality is on either the α- or the β-chain.

Neel: Is the naming of the peptides by numerical designation entirely arbitrary?

Ingram. It is purely arbitrary numbering, derived from the actual fingerprint It has no significance in terms of ordering the peptides We don't know the order, except for peptide No. 4, which is the beginning of the β-chain

Lederberg: I would like to ask the group to give us a consensus on the question of the homogeneity and uniformity of the primary structure of proteins as they appear in nature. We have all quoted Sanger's work as showing that there is a unique structure for insulin as it is naturally produced But it frequently appears as though there were faint spots in the background of the peptide fingerprints What limit would you set on the degree of valid, not spurious, variability in the actual composition of the protein?

Ingram: I think I would say that all such variability can be laid entirely to imperfect digestion of the protein

Lederberg: How much is attributed to this source and how much to variability in the original composition?

Anfinsen: In some cases, it can be said that the error must be very small if it exists at all. For example, in ACTH, one group could add up all the components in various kinds of digests to levels well above 95 per cent (89). In ribonuclease, recovery is 100 per cent for many peptides (39)

Spiegelman: This is a very insensitive way to get an answer to that question

Lederberg: You have mentioned that there is this background of material which you don't include in your analysis or in your specification, and I would like to know how much of this could be lurking in the background.

Anfinsen: As someone said, in the case of hemoglobin, the presence of a tremendous number of peptide components in the fingerprints, of the order of 1 to 3 or 4 per cent, can't be ruled out

Ingram: We already know that in the hemoglobins, there are about a half dozen small, minor components present

Lederberg: But the protein that you separate and call hemoglobin A is what you are analyzing

Ingram: No, the protein of which I have shown the analysis is the whole, with the minor components present; the minor components do not show up in the peptide analysis

Neel: This leads to a question with respect to some of the hormones and enzymes There is excellent evidence that for some enzymes there is an invariate part, the same in every species studied to date However, the "tail" of the enzyme may vary from species to species If there are these interspecific differences, there is certainly no reason to think that there are not intraspecific differences in the tail To what extent have the analyses shown that?

Anfinsen: Analyses have been made, in our own experiments, on both single and pooled specimens In other words, we have studied 200 eggs and one egg, and we have studied 15 pancreases and one pancreas In either case, we get the same results

Ingram: It is useful to have a shorthand method for indicating the molecular constitution of these hemoglobins A method I have used is shown in Table I We have talked about there being two α-chains, so we write α_2 In hemoglobin A, these are normal, so it is $\alpha^A{}_2$ There are also two β-chains, so it is $\beta^A{}_2$ Abnormal peptide chains are indicated by the appropriate superscript

The *genetic* constitution of individuals with abnormal hemoglobins can also be indicated with related symbols. If α and β represent the two loci controlling synthesis of the α- and β-chains, a normal individual with hemoglobin A would be designated α^A/α^A, β^A/β^A An individual homozygous for hemoglobin S would be α^A/α^A, β^S/β^S, and an individual heterozygous for hemoglobin S would be α^A/α^A, β^A/β^S For hemoglobin I, the designation for the heterozygote, the only form known,

TABLE I

Designations of Molecular Constitution of Hemoglobins

Hemoglobin Type	Constitution
A	$\alpha^A{}_2\beta^A{}_2$
S	$\alpha^A{}_2\beta^S{}_2$
I	$\alpha^I{}_2\beta^A{}_2$

would be α^I/α^A, β^A/β^A Different individuals use slightly different forms of this

Motulsky · I am not happy about using the superscript A for the normal hemoglobin such as $\alpha^A{}_2/\beta^A{}_2$ The α-chain of fetal hemoglobin presumably is identical to the α-chain of adult hemoglobin In the nomenclature used by Dr Ingram, fetal hemoglobin would be $\alpha^F{}_2/\gamma^F{}_2$ This designation might suggest that the α^F chain is different from the α^A chain of the normal adult hemoglobin; actually they are identical I would therefore suggest using the superscript $^+$ when dealing with the normal "wild type" hemoglobin phenotype or genotype. This nomenclature is outlined in Figure 19.

Ingram. This is a point that could be discussed when hemoglobin and thalassemia are considered It is purely a point of nomenclature, as long as we know what we're talking about

This leads us directly to the whole problem of the relationship between the structure of the gene and the structure of the polypeptide chain, between the fine structure of the gene and the amino acid sequence of the peptide chain. Dr Levinthal can tell us about his studies on alkaline phosphatase and also the way information might be transferred, or the coding problem

Levinthal · There are a number of investigators working on systems in microorganisms in the hope of doing both fine structure genetics and protein chemistry of the kind that Dr. Ingram has been discussing I think it is fair to say that at the moment the chemistry has not been carried to anything like the point it has reached in the case of hemoglobin in these microbial systems On the other hand, with respect to the genetic analysis, it has been carried considerably further

Hb LOCI

$$^{\alpha}\theta\ \ \theta^{\alpha}\ \ {}_{\beta}\theta\ \ \theta_{\beta}\ \ {}^{\gamma}\theta\ \ \theta^{\gamma}$$

	GENE NOTATION	GENE PRODUCT(S)
Normal man	$Hb\alpha^{+}/Hb\alpha^{+}$ $Hb\beta^{+}/Hb\beta^{+}$ $(Hb\gamma^{+}/Hb\gamma^{+})$	$\alpha_2^{+}\beta_2^{+}$ (Hb A) $[\alpha_2^{+}\gamma_2^{+}$ (Hb F)$]$
Sickle trait carrier	$Hb\alpha^{+}/Hb\alpha^{+}$ $Hb\beta^{+}/Hb\beta^{S}$	$\alpha_2^{+}\beta_2^{+}$(Hb A)* $\alpha_2^{+}\beta_2^{S}$(Hb S)
Sickle cell anemia	$Hb\alpha^{+}/Hb\alpha^{+}$ $Hb\beta^{S}/Hb\beta^{S}$	$\alpha_2^{+}\beta_2^{S}$ (Hb S)
Hb I carrier	$Hb\alpha^{I}/Hb\alpha^{+}$ $Hb\beta^{+}/Hb\beta^{+}$	$\alpha_2^{+}\beta_2^{+}$(Hb A)** $\alpha_2^{I}\beta_2^{+}$(Hb I)
Double heterozygote S & I	$Hb\alpha^{I}/Hb\alpha^{+}$ $Hb\beta^{+}/Hb\beta^{S}$	$\alpha_2^{I}\beta_2^{+}$ (Hb I) $\alpha_2^{+}\beta_2^{S}$ (Hb S) $\alpha_2^{+}\beta_2^{+}$ (Hb A) $\alpha_2^{I}\beta_2^{S}$ (Hybrid S/I)

* not $\alpha_2^{+}\beta^{+}\beta^{S}$
** not $\alpha^{+}\alpha^{I}\beta_2^{+}$

FIGURE 19 Suggested notation for hemoglobin genes and gene products

I will review briefly the status of the work with alkaline phosphatase being done by Alan Garen, Frank Rothman, and me. We have been working with the enzyme alkaline phosphatase of *E coli*. The enzyme cleaves rather nonspecifically the phosphate ester linkage of various organic phosphate compounds The function of the enzyme in the cell is somewhat dubious But one of its functions at least is to supply the organism with a source of phosphorus if it is in a medium in which the only phosphorus is in the form of organic esters. A cell with the enzyme can utilize this source of phosphorus whereas a mutant which lacks the

enzyme cannot use organic phosphate as the only source of phosphorus It is this characteristic which we make use of in carrying out selective genetic analysis The control mechanisms which govern the rate of production of alkaline phosphatase in *E. coli* were worked out independently in two laboratories Torriani (99) and Horiuchi and co-workers (41) found that the enzyme is produced in high concentration if the cell is grown in the absence of any source of phosphorus.

Davis· Do you mean in the absence of inorganic phosphate?

Levinthal: No, I mean in the absence of a source of phosphorus

Lederberg· How do you grow it?

Levinthal· The cell is grown under conditions of phosphate limitation The organism grows logarithmically, up to the point where the phosphorus in the medium is exhausted From this point on, there is considerable increase in the cell mass, in the cell protein, and also in cell numbers In addition, during this time, DNA increases slightly, protein increases considerably, RNA decreases somewhat, and alkaline phosphatase is made in high concentration

Of the new protein that is formed, about 7 per cent is alkaline phosphatase (31) This is comparable to the amount of β-galactosidase formed under optimal conditions of induction. And it also seems to hold for several other bacterial enzymes It seems to be a reasonable generalization that under conditions in which the bacterial enzyme is made at its maximum rate, that is, either full induction or complete release of repression (which are probably the same things as we will hear later in the discussion), the particular enzyme can probably be made to the extent of 5 to 7 per cent of the total cell mass This means that any protein for which one knows the conditions required to release repression is relatively easy to purify

Purified phosphatase has a molecular weight of approximately 80,000, and from recent experiments of Rothman we have reason to believe that it is made of two identical subunits The evidence is of two kinds: First, by using urea and agents which break disulfide bonds, components which have a molecular weight of 40,000 can be extracted. Furthermore, amino acid analysis of the total protein has been done, and from the number of arginine and lysine residues contained in the protein and from the number of spots which contain arginine and lysine on the peptide fingerprint, the effective size of the primary unit can be deduced This is found to be 40,000.

Now I would like to say something about the genetics of the phosphatase system. The enzyme was originally chosen for this work for two reasons: because the physiology of the repression was under-

stood and because it was an easy enzyme to assay. There is a readily available substance, *p*-nitrophenylphosphate (PNPP), which is colorless and which becomes colored when the phosphate is cleaved

The organisms can be grown on Petri dishes with limiting phosphate Under these conditions, the colonies all grow to approximately the same size, and those cells which can make the enzyme produce it at high concentrations. If these plates are sprayed with PNPP, the colonies which have made the enzyme turn yellow, and those which have not made the enzyme do not turn yellow. By irradiating cells and looking at 10,000 to 20,000 of them, mutants which fail to make the enzyme can be selected Organisms which have no detectable enzymatic activity can be selected by this test as can those which have a reduced amount of enzymatic activity Both of these kinds have been found

The genetics has been done primarily with the kind which has no enzymatic activity These mutants are also unable to grow on glycerol phosphate as the only source of phosphorus, whereas the wild type and the leaky mutants are able to grow on glycerol phosphate Under these conditions, then, there is a selection system, because it is possible to plate large numbers of mutant cells which will not grow in glycerol phosphate In a recombination experiment, a plate which contains *glycerol phosphate as the only source of phosphorus is used.* Under these conditions, only the wild-type recombinant cells and the revertants will grow

It is possible either to select revertants or measure reversion frequency. The sensitivity of this test is somewhat better than 1 part in 10^7, that is, one wild-type cell can easily be selected in the presence of 10^7 mutant cells

These mutants have all been obtained in *E coli* K12, the Cavalli high-frequency recombination strain, which can be designated as HfrC.

I will have to say a little about recombination in bacteria (56) When a high-frequency recombination (Hfr) male cell is mated with an F^- female cell, a cytoplasmic bridge is formed between these two, and the chromosome of the male cell passes into the female in a way which is well defined both as to time and orientation. The head of the chromosome goes in first, and it takes approximately 1 hour for the whole chromosome to get across The phosphatase mutants are located between *lac* and *T6* on the bacterial chromosome

If an F^- cell, which is phosphatase-plus, is mated with an Hfr cell, which is phosphatase-negative, the time at which the phosphatase-negative gene has entered the F^- cells can be determined, because from that time on some of the F^- cells will produce among their progeny a

few organisms which lack the ability to cleave PNPP. We can thus obtain F^- cells which have the phosphatase-negative gene

For most of the P^- mutations, when such a cross is done, the F^- progeny are either totally P^+ as the original F^- parent or totally P^- as the original male parent However, occasionally one finds F^- cells, which have a partial enzymatic activity We think that the mutant gene is unable to express itself in the environment of the Hfr cell but it is able to express itself partly in the environment of the F^- cell

Fremont-Smith Are you implying that there are two P^- genes, one of which is absolute and one of which is only partial and in the appropriate environment can produce enzyme?

Levinthal: I am implying that there are many P^- mutations which could occur in the same gene Some of these produce a total absence of the enzymatic activity and some produce altered enzymes in the Hfr, and some produce no enzymatic activity in the Hfr and an altered enzyme in the F^-

Lederberg. We have found this to be the case with quite a few sugar fermentations There were some F^- stocks that accumulated many modifiers which wouldn't be known about until mutant genes had been introduced into those backgrounds Then it was evident that they gave rise to intermediate means of expression

Levinthal As far as the proper genetic analysis is concerned, this has been done only with those P^- mutations which behave as total negatives in both the Hfr and the F^- Fourteen of these mutants have been crossed by Garen (30) in many combinations A reasonably additive map has been obtained in this way in which the total recombination frequency between the two extreme P^- mutants is about 0 2 per cent The minimum distance for any pair examined so far is about 0 01 per cent The first point to notice here is that all the mutations which affect this particular enzyme are closely linked to each other. If we define the word gene as a localized region of a genetic map in which mutations affect a particular protein, all of the phosphatase mutants lie in a single gene We have not done the cis/trans analysis yet, so we cannot use the word "cistron"

We can now make a rough estimate of the size of the gene in chemical terms Fuerst, Jacob, and Wollman (56) labeled Hfr cells with P^{32} and then stored the cells frozen and allowed the P^{32} to decay, prior to thawing and mating them with suitable F^- cells. They found that the probability with which the chromosome was ruptured, preventing transfer of particular markers to the F^- cell, was proportional to the distance of these markers from the origin of the chromosome and

the specific activity of the P^{32} which had been introduced into the Hfr cell. They concluded, therefore, that a certain fraction of the P^{32} decays caused a rupture of the chromosome, preventing the transfer of the genetic region beyond this rupture They also concluded that, at least to a first approximation, there was a constant amount of P^{32}-containing material per unit length of chromosome, where chromosome is now defined in genetic terms

The total amount of DNA in a bacterial nucleus can be estimated, and therefore the total amount of DNA which is transferred in such a mating experiment can also be estimated These estimates are rough, but they are probably correct to within a factor of 2 The total amount of time necessary to transfer the DNA can also be measured and therefore the amount of DNA transferred per unit time can be deduced in terms of P^{32} atoms per minute

Garen has recently done such experiments and by means of them can estimate the amount of DNA between two phosphatase mutants as compared with the amount of DNA between two marker mutants, where the measurement can be made more directly In doing that, the size of the gene turns out to be of the order of 2000 nucleotide pairs

Lederberg In other words, 0 18 per cent

Levinthal: Yes, 0 18 per cent corrected for the number of mutants used This was actually done with 14 mutants We assume they are randomly distributed along the gene This would give a total gene recombination frequency from which a total amount of DNA can be inferred

Since the half-unit of the phosphatase molecule contains about 400 amino acids, this sequence is the information which must be controlled by the phosphatase gene As a first approximation, then, the mean number of nucleotide pairs per amino acid is of the order of 5

The first question, of course, in evaluating how reliable this value of the coding ratio is, is what sort of experimental deviations can be expected It is reasonably safe to say that the coding ratio obtained from our data is reliable to within a factor of 2 The most important thing about these results is that the coding ratio is small Prior to this, the best estimate available was from the tobacco mosaic virus, where it was known that the RNA controlled at least the protein of the virus and probably much more From that alone, it could be said that the coding ratio for single-stranded RNA would have to be a maximum of 40 or 50 to 1.

Aside from the possibility of experimental error, there are also, of course, possible errors of fundamental theory, which could make this

estimate totally meaningless I would like now to talk about the assumptions that have gone into this, and the way in which parts of the "dogma" are implicit in these assumptions.

Fremont-Smith· Are you going to state the "dogma" at this time?

Levinthal: Yes First, we know that there is a genetic map and, along this genetic map, mutations can be arranged in a linear sequence Operationally, the linearity of this sequence has nothing to do with any physical entity It comes out of recombination experiments of various kinds and is deduced entirely from the probabilities of obtaining various progeny out of crosses The one-dimensionality of the genetic map applies both to the arrangement of mutations between genes and the arrangement within genes.

In addition, we know that DNA carries the genetic information and that it is essentially one-dimensional in the sense that the information is presumably contained in the order of the bases along the molecule

Item 1 of the dogma is that there is a 1 to 1 correspondence between position on the genetic map and position along the polynucleotide chain of the DNA Furthermore, it is assumed that the order and the relative distances of mutations along the genetic map is a direct reflection of the order and relative distances of alterations in the polynucleotide chain

These assumptions might be in error in the following ways· There might be no correspondence at all between the genetic map and the DNA molecule, there might be a 1 to 1 correspondence between mutations and alterations in the DNA but there might then be some reason why the orders are not collinear on the two; and there is a modification which seems quite likely, namely, that although the relative order is preserved between the genetic map and the DNA molecule, relative distances are not This would be the case if recombination were more frequent in some regions of the DNA than in others.

The second item of the "dogma" is that DNA controls the primary structure of proteins The reasons for acceptance of this point differ considerably In general, the determining factors are when the individual entered the field and what he was thinking of at the time. The only experimental facts relevant to this are those mentioned by Dr. Ingram, namely, that some mutations do affect the primary structure of proteins However, this fact by itself is certainly not enough to establish the "dogma." I feel that the most convincing fact is that the gene is a one-dimensional structure and the only evident thing about a protein which is one-dimensional is the primary sequence of the amino acids

along the peptide chain However, this is certainly not a very strong argument and therefore we need some experimental data.

Lederberg: I can't quite accept that The information needed to specify both the primary and the secondary structure of a protein could be written on a single linear tape The bond angle to the next residue could be written after each amino acid specification.

Levinthal: The reason we call this "dogma" is that it depends on personal bias, not logic.

Lederberg: I agree with you on biases, but if the main element of this bias is a linear chromosome, a linear instruction could be written which would include explicit specifications for the secondary structure

Levinthal. Perhaps it is unnecessary to go into the reasons why we want to accept these assumptions This is what we are accepting as the hypothesis, to be tested in an experiment. The next point of the "dogma" is that once a primary sequence of a protein has been formed, the sequence cannot be changed by any rearrangement of the amino acids. Of course, this does not preclude cleavage reactions, which we know to exist, for example, in the formation of trypsin from trypsinogen However, we do not think that a sequence of amino acids in the polypeptide chain is altered once it has been formed.

Anfinsen In the case of trypsinogen and chymotrypsinogen, the active enzyme lacks the seryl-arginine bridge while the inactive proenzyme has this bridge In terms of genetic information, the information must have been present first for the active enzyme This is on purely teleological grounds The sequestering must have come later. It would be very difficult to imagine "natural selection for intention," and that is what the reverse situation would be. A way must be found, in terms of the long run of biology, to add this small bridge at a later time.

Levinthal: I am very suspicious of any argument which is based on evolution I am sure that whatever we find, we could all make up good reasons to explain its selection in nature.

Lederberg Except a change in the code.

Levinthal. No, even a change in the code. These ideas are basically what we call a "dogma"

Fremont-Smith Is any one person responsible for this basic "dogma"?

Levinthal: I feel that it has come about through the conversations of many people

Fremont-Smith There is no one place where it is stated in the literature?

Levinthal: Crick (19) stated it very well in his article on protein

synthesis, and it was he who first called these ideas the "dogma," but they have certainly been around since 1953, at least, and I assume they were probably around before then

Anfinsen. Caspersson, in 1939, more or less suggested this (15).

Levinthal: I'll review the coding situation briefly. DNA, which contains four bases, is treated as a four-letter alphabet that must determine the sequence of twenty amino acids. Obviously, one nucleotide can't code for one amino acid. A set of two nucleotides would give only sixteen combinations, which would not be enough to code for the twenty amino acids Therefore, a three-letter code would appear to be the smallest one possible Various kinds of three-letter codes have been discussed The one that I find most attractive is that of Crick, Griffith, and Orgel (20), the so-called three-letter "commaless code" If the nucleotides are designed by the letters A, T, G, and C, words of three letters can be written and the commaless code is defined in the following way:

There are certain combinations of three letters which correspond to particular amino acids. For example, ATG might correspond to lysine. Some other three-letter sequence would correspond to a different amino acid. The assumption of the commaless code is that if two such words are written next to each other along the chain, for example, ATGCAT, corresponding to lysine-valine, then TGC, which occurs as the overlap between them, must not correspond to any amino acid or, as it has been expressed, must not be in the dictionary Likewise, GCA must correspond to no amino acid. Since, presumably, lysine might follow valine somewhere else in the chain, which would mean a sequence CATATG, then TAT must not appear in the dictionary It turns out that a code of this type can be constructed using four letters taken three at a time which is made up of twenty, and only twenty, words. Since twenty is the number of amino acids, this has some relevance The mathematical problem of writing down all possible codes of this kind has been solved by Freudenthal (28) and by Golomb, Welch and Delbruck (33).

In any code of this type, there exist words which are not in the dictionary, and one might ask what these nonsense words would do if they occurred in the DNA. Suppose, for example, a mutation occurred in a sequence which led to replacement of CAT by TAT, which, as indicated above, must not be in the dictionary. Interpreting this literally, we would find that in the amino acid sequence there would suddenly be a gap This kind of nonsense mutation might have various effects, but it is not unreasonable to expect it to cause the total absence of the particular protein involved

There could also be mis-sense mutations, that is, mutations which change one dictionary word into another These are presumably the kind that Dr Ingram talked about in hemoglobin A mis-sense mutation, which causes one amino acid to be replaced by another, will have more or less effect on the function of the protein, depending on where it occurs in the molecule If such an alteration occurs in the active site of an enzyme, it should be more important than if it occurs on a tail somewhere If the alteration produced a change of charge, it might be more important than if a similar amino acid were introduced with no charge changes On the other hand, a nonsense mutation would be expected to cause a functional change regardless of where it occurred in the molecule.

Experimentally, of course, it may be very difficult to distinguish between these two types of mutations, since a mis-sense mutation may prevent the polypeptide chain from folding in a way which resembles the normal protein However, I would like to mention, with considerable reservation, one experimental point relevant to this When there is a random change of one letter for another, what will the probability be that this change produces a mis-sense mutation and what will be the probability that it produces a nonsense mutation? The answer is that about half of the mutations will be nonsense and about half mis-sense, and it has turned out experimentally in several systems that approximately half of the mutants which lack enzymatic activity do make an immunologically cross-reacting material and about half of them do not However, there are a great many reasons why there may be no real relationship between this experimental fact and the theory

Spuhler Why shouldn't that ratio be about 1 to 2, since there are sixty-four possibilities?

Levinthal. Because not all sixty-four can be obtained by single-step mutations It is instructive to experiment by writing codes There are some words that can't be reached by one-step mutations

Ingram Isn't there also a restriction as to the number of mis-senses?

Levinthal Analysis of the possible amino acid changes which could be produced by various single-step mutations can become quite elaborate If a great mass of data were available on the amino acid changes produced by single-step mutations, it should be possible to gain some insight into the nature of the code However, as Dr Ingram said earlier, this has so far only been done for four mutations, which may or may not be single steps

Atwood Wouldn't it be simpler just to look for the neighbor restrictions in degraded proteins and see what can be found?

Levinthal: No, the "commaless codes" do not imply any neighbor restrictions in adjacent amino acids. In fact, they were formulated because the previous codes proposed by Gamow (29) implied restrictions of neighbors in the protein. Brenner (12) has analyzed the known sequences and has shown that the sequences available already rule out the kinds of neighbor restrictions which resulted from the Gamow type codes

Neel. Even if all possible neighbor combinations are found, this doesn't necessarily prove that any amino acid can get to any other amino acid in one step Nothing is known about intermediate steps.

Ingram. In the hemoglobins, there is absolutely no evidence that these mutations are one-step mutations We just don't know

Levinthal· This is true However, in the case of microorganisms, it is possible, by using chemical mutagens at very low concentrations, to ensure that a single-step mutation is being dealt with.

Atwood· Since the actual letters of the code are base pairs rather than bases, we must choose one of the strands arbitrarily and then say, "Now, we go down this one" This takes us back to the problem of inversion It is evident that if an inversion is made, the strands must be transposed. Therefore, the code becomes completely different throughout the inverted segment with respect to the uninverted. We don't notice any such drastic effects. Sometimes, an inversion is associated with mutation at or near a break point, but disturbance of genetic function throughout a long inverted region is not observed

Levinthal The fact that DNA is a double helix certainly does bring up the problem of how one knows which strand to read in forming a protein Two types of solution have been proposed for this difficulty. One is to make up a code which will only make sense when read along one chain and will always make nonsense when read along the other chain This type of code would require more than three letters; however, with a more complicated code, it could obviously be done.

Atwood: Again, the inversion would have a definite mutating effect

Levinthal: No, it would not produce a mutation, unless the edge of the inversion occurred within a gene However, there is another alternative which also can eliminate this difficulty. Let us imagine that there are words which correspond to amino acids and sentences which correspond to polypeptide chains. Between sentences, as punctuation marks, there might be a sequence of nonsense words which could determine which of the two chains would be read. For example, if there were a sequence AAAA on one chain of the DNA, corresponding to the se-

quence TTTT on the other chain, this information could be used to determine which of the two chains was to be read in making RNA There could be a rule requiring the production of the RNA templates only after the sequence of A's This would not interfere with the fact that the commaless code would increase the efficiency with which protein could be made on the RNA template.

Atwood. In other words, the inversion problem is avoided if it is assumed that the reading switches from one chain to the other at each break point

Lederberg: To state a point of personal bias, I have sometimes felt that the commaless code was clever but implausible and unnecessary We are certainly not compelled to think that biological expression is so terse, when we see how extensively redundancy is practiced for security There may be rather explicit commas, special sequences (monotonous doublets or triplets?) that mark off the unit words The commas would also deal with the transposition problem, that is, which of the complementary chains should be read Finally, commas may also act as spacers so that the DNA may even have some instructions for the folding of the protein as it is made

Spiegelman: The only reason for the commaless code, actually, is in the next step There should not be too long distances in the RNA template.

Marks: Do the observations with regard to "hot spots" modify the conception of the code?

Levinthal No, the hot spots refer to the fact that a particular transition, let's say the transition AT to some other nucleotide pair, occurs with a higher probability if it has AT and GC around it than if it had some other neighbors around it But this has to do with the theory of mutations, which is another topic

Neel. An inversion, by and large, would be associated with a change in the content of the code I would like to ask those here who are familiar with *Drosophila* what proportion of inversions—and there have been studies along these lines—has been shown to be associated with detectable mutations?

Lederberg: Or how many homozygous inversions in *Drosophila* are completely normal in phenotype?

Stern· I think the majority are lethal when homozygous Isn't that true?

Lederberg That would be my impression

Stern: But I don't know the number

Atwood: The stock explanation for that is that most of the inversions

arise from irradiation, and you have knocked out a little segment at one of the break points Sometimes, this can be shown to be true, but not always.

Steinberg: Gruneberg's case of *roughest-3* inversion ties in well with this (34). It is a nice case in which the inversion led to a detectable change, and with the reinversion it was lost

Ingram. Figure 20 illustrates the triplet of Crick, Griffith, and Orgel (20) as applied to the sickle-cell mutation, just purely schematically, of course, picking out three-letter words for the particular amino acid. Imagine that one base pair changes according to the rules of that particular code This is the kind of picture obtained. You can, of course, extend it to take in mutation to hemoglobin C by changing the same original T-A base pair, which would give lysine.

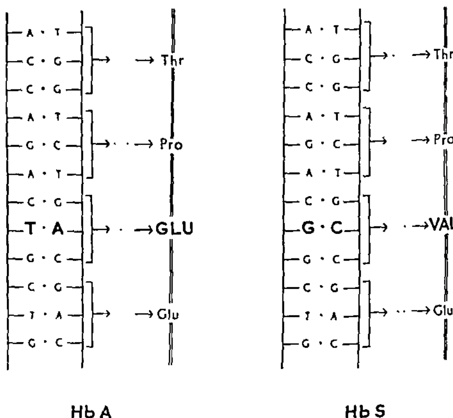


FIGURE 20 Schematic representation of changes in the triplet code for DNA affecting the amino sequence in peptide No 4 of hemoglobins A and S

Mourant (70) suggested that the hemoglobin C mutation might have arisen from the hemoglobin S mutation, the basis of this suggestion being the occurrence of hemoglobin C Its mutation seems to be confined to one restricted area in Ghana, where hemoglobin S is high and where hemoglobin C seems to be spreading from one locus

Neel: At the moment, it cannot be said with certainty whether or not the hemoglobin C mutation arose in a high S population There are a number of small scattered tribes in northeastern Ghana and in the Upper Volta region of French West Africa which have not been at all well studied It is very difficult to say whether the maximum concentration of hemoglobin C is found in tribes that do not have S or whether it is the picture of hemoglobin C displacing S in tribes which had S

Ingram: Here, at least, is a suggestion of a two-step mutation In all other cases, there is not even a suggestion that the particular hemoglobin mutation is a one-step mutation

Neel: If it is assumed that in the beginning hemoglobin was a fairly homogeneous substance from individual to individual, the one-step, two-step, three-step mutation hypothesis would imply that some amino acid changes are much more common than others Is there a hierarchy of frequencies that might be built up here?

Lederberg We can't discuss the mutational changes in hemoglobin, because we don't have any evidence that hemoglobin A is the ancestral hemoglobin in man

Neel: Let's go back to alkaline phosphatase Is there a hierarchy of frequencies which would be expected from this concept of the code?

Levinthal No, there is not

Lederberg The most comprehensive study that relates to this is Benzer's study of mutation rates (9) He gets such a wide range of different mutation rates that we cannot possibly attribute a given mutation rate to a given singlet, doublet, or triplet There are too many mutation rates to be able to do this

Fremont-Smith: Is there any possibility that the fetal hemoglobin is closer to the primitive hemoglobin than the adult hemoglobin is, and is there any relationship of fetal hemoglobin in man to fetal hemoglobin in any of the other primates?

Ingram We don't know There is no reason to suppose that fetal hemoglobin is more primitive than adult hemoglobin, and there is no chemical evidence relating the structure of human fetal hemoglobin to the various animal fetal hemoglobins.

Fremont-Smith. Isn't there reason to believe phylogenetically that the

fetal hemoglobin is more primitive? This is true of most organ systems in the body

Ingram. Yes, but I don't think there is any chemical evidence to back it up.

Swinyard: Have the biochemical studies of fetal hemoglobin been related in any way to the megaloblastic or normoblastic generations of fetal blood development?

Motulsky: I don't think they have

Levinthal: I would like to ask a question of the anthropologists present In the case of bacteria, it can be said that in a population of 10^9 cells which arose from one cell, an estimate can be made of the mutation frequency in a given protein There are approximately 10^9 human beings on earth If the question is now asked, how many cell generations, how many chromosomal generations or nuclear generations are involved since these 10^9 organisms diverged from a few organisms, what sort of estimate do you arrive at? What orders of magnitude? Is that an answerable question?

Lederberg: Do you mean during the development of the organism or during evolution?

Levinthal: I mean the two multiplied together

Spuhler: I once figured the average number of cell generations in man In the female, it's something like 50. In the male, correcting for loss of sperm and manufacture of sperm over some time, the calculation was 70 or 80

Neel This is from zygote to sperm

Levinthal We have a number of 50 per generation How many generations of people are there?

Stern It would take perhaps 500,000 years. That would give, at four generations per century, something like 20,000 successions.

Neel: So if 20,000 is multiplied by 50, we obtain 1,000,000 cell divisions, plus or minus, in the history of the human race Is this the figure you're trying for, Dr. Levinthal?

Levinthal Yes, in other words, we would expect, if we looked at, say, the most distantly related humans around now, they might have diverged, or their genes might have diverged, from a common ancestor something like 1,000,000 cell generations ago.

Neel. This is possible.

Levinthal: I wonder whether the anthropological data could reduce this number in a significant way.

Neel: There is the problem of selection Without selection, with

this figure of 1,000,000 and an average mutation rate of 1 in 100,000, difficulties arise in understanding how the members of the human species could still apparently have so many genes in common.

Levinthal: That's why I think this number of 1,000,000 is too high.

Neel: We are overlooking selection in this whole argument.

Steinberg: I think the number is too high because we have taken the number of divisions for the entire organism and what we're interested in is divisions from the fertilized egg, the establishment of the gamete, to the gonad, which is many fewer. All the other divisions go into the formation of the soma and are not pertinent to the discussion.

Neel: But we would have to include the divisions of spermatogonia, wouldn't we, within the seminiferous tubule?

Lederberg: Nothing of these calculations is going to be accurate within a factor of 10, anyway, and that's what we're talking about. What we find is that every human locus since the start of the species has an expectation of mutation of about one, which is quite compatible with the observed fact. It takes very little selection to have driven off the rare mutants. If it were a completely neutral situation, if we were discussing just mutation pressure and nothing else, if there were absolutely no selection whatsoever, it would be expected that roughly half of the loci would differ from the loci of related organisms.

Levinthal: I am thinking about the experiments Dr. Ingram described in normal individuals, in which he considered mutations which produce amino acid changes with no physiological effect on the hemoglobin function. With what probability do we expect to find amino acid variations in the population?

Lederberg: If there were no selection, approximately 1, or something like that.

Neel: Some of the earlier conversations between Dr. Ingram and me were on the idea that the probability, barring selection, is very high. If one were to analyze 100,000 specimens of hemoglobin and find only a very limited number of changes, this would give a great respect for selection.

Levinthal: In this whole discussion we have neglected the kind of thing that Sewall Wright looked at. I mean not the selection of the hemoglobin gene but the selection of other genes, which essentially put one through a bottleneck at various stages. I was wondering what the most recent bottleneck was.

Spuhler: There is another factor, though. I don't think it is possible to get a good estimate by just taking the line we have taken here. In a population where sibship size has a Poisson distribution, the probability

of any one mutation surviving is e^{-1}. If these populations are quite small, as they must be in the history of mankind, and if a beginning is made back about 1,000,000 years ago with some nonhuman primate ancestor, the effective size is certainly not greater than the order of 500 or so So mutation pressure alone for a mutant that is completely neutral would not build up this frequency very high.

Lederberg: No, I think the expectations will remain exactly what they were, but you're getting into the jackpot phenomenon, and so on. Any hereditary stochastic process has a higher than Poisson variance

Ingram. By the end of this conference series we will have some data on it which will give some degree of confidence.

Lederberg: It would be interesting to get fingerprinting on some of the more nearly related primates.

Atwood: Is it true that every mutation where no protein at all is produced is unsuppressible, so far?

Levinthal. Yanofsky (106) published a paper in which he reported a mutant which produced no CRM but which was suppressible So far, in *E coli*, we have found no suppressor I am quite prepared to accept the theory that these suppressors will turn out to be concerned with inhibitors and factors which affect inducibility or suppressibility of the enzyme

Davis. I can't see that the production or nonproduction of a cross-reacting protein can be considered very fundamental In a case of suppression that has been nicely analyzed by Susskind, the suppressor restores enzymatic activity in the cross-reacting protein by decreasing the uptake by the cell of zinc ions, to which this protein is excessively sensitive However, other mechanisms of suppression are also quite possible Thus, we know many normal control mechanisms in which the production of an enzyme can be blocked by the addition of an appropriate small molecule, which we call a repressor It is easy to imagine a mutation that blocked production of an enzyme by increasing the sensitivity of the system to an endogenous repressor A suppressor mutation could reverse this effect by interfering with formation of the repressor.

Ingram: I think that this discussion of the suppressor mutations in the enzyme systems is relevant also to the hemoglobin system, particularly to the thalassemia diseases, where suppressor mutations have been invoked by some people.

Lederberg: There is one way this repressor could work. Imagine a mis-sense mutation with no demonstrable cross-reacting material. This is a mis-sense of a kind that interferes with the inducibility, with the

removal of a protein from the template. Suppressor mutations could then change the cellular environment to make possible this removal, to make it inducible.

This is why all the stress should be placed on finding what the primary structure of the product is. It is not important to ask whether the demonstrable material is formed or even whether it is enzymatically active, because here it may very well be that extra materials are being dealt with There is no conclusive information yet in this system or in any other suppressor system concerning primary structure

Fremont-Smith: Is it fair to say that the "dogma" as now stated is the simplest concept which is flexible enough to take into account the main or all the facts that are now available? It will be interesting to see, over the 5-year span of this series of conferences, what happens to the dogma, at which point it gets ruptured first

Ingram: We have discussed DNA and we have discussed the final protein structure, but so far we have largely ignored the chemical steps between It would be appropriate now to have Dr Hoagland give some recent ideas on that subject

Hoagland: An important aspect of the intermediate steps of protein synthesis, particularly as far as problems of coding are concerned, is the clear demonstration that RNA is involved directly in the process We know now that the major synthesis of protein occurs in the ribosomes The RNA of these ribosomes is of relatively high molecular weight, consistent in size with what one might expect would be required for a template for proteins of average molecular weight

A great deal of work has gone into a study of the mechanism of protein synthesis, and Figure 21 summarizes what we think we know about the mechanism Amino acids initially have to be activated before they can participate in protein synthesis We are convinced now that this occurs by the mechanism shown, in which enzymes specific for each of the twenty amino acids (there being twenty such enzymes) react with their specific amino acid to form amino acyl adenylates These adenylates are firmly bound to their respective activating enzymes Then, a very interesting thing seems to occur There is a particular species of RNA of the cell which has been called soluble RNA because it remains in solution when the ribonucleoprotein particles are spun down This SRNA, or transfer RNA, reacts with the amino acyl adenylate-enzyme complex with the result that the amino acid is transferred to the RNA

Amino acids become attached to the end of the RNA chain, the amino acid carboxyl group being linked to the 2'- or 3'-hydroxyl of the ter-

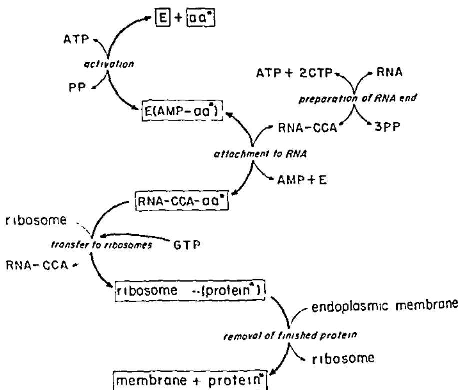


FIGURE 21 Steps in protein synthesis

minal adenosine moiety of the transfer RNA. Then apparently the transfer RNA bearing the amino acid enters the ribonucleoprotein particles, and there the amino acid is next found accumulating in protein

A great deal of this work has been carried out by the use of radioactive tracers, starting out with a free radioactive amino acid and finding that it appears in protein The question of whether true synthesis of protein is occurring in such systems is a very important one Recently, Schweet (87) has done a very nice experiment with reticulocytes, in which he has shown that hemoglobin actually does accumulate when one incubates the cell-free system with these various components It is still an extremely crude system, and the only point I wish to make about Schweet's experiment is that it is one of the few demonstrations in a dissected system of this sort that protein is actually being synthesized, and it is consistent with the earlier results using radioactive amino acid incorporation into mixed cell proteins

The aspect of our scheme that has a bearing on the coding question is the matter of the direct reaction of an activated amino acid with soluble RNA Figure 22 shows a theoretical scheme which we proposed to account for the reason why amino acid may initially react with soluble RNA before it enters peptide linkage of protein

Before the discovery of soluble RNA, Francis Crick suggested that it was very difficult to conceive how one of these twenty different amino acids, as a chemical entity, could "recognize" one of the triplets on the template. Just on chemical grounds, it did not seem feasible that valine would "know" how to get to the triplet C-A-U, for example He suggested that the amino acid might first react with a triplet of nucleotides complementary to the triplet in the RNA strand For example, valine would react first with an "adaptor" triplet with a coding sequence G-U-A that would be available to the activating enzyme in the soluble milieu

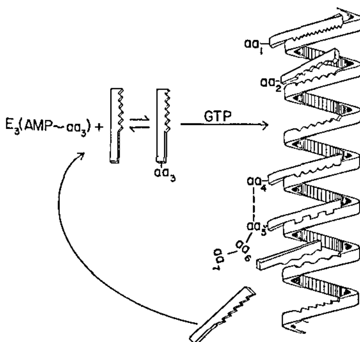


FIGURE 22 A scheme for the interaction of microsomal RNA and soluble RNA-amino acid Reprinted, by permission, from Hoagland, M B, Zamechik, P C, and Stephenson, M L A hypothesis concerning the roles of particulate and soluble ribonucleic acids in protein synthesis In *A Symposium on Molecular Biology*. R E Zirkle, Editor Chicago, Univ of Chicago Press, 1959 (*pp 105-114*)

bathing these particles Then, the adaptor would find its way to the template and would line up in a complementary position with valine projecting out In this way, a series of amino acids could line up along the template and their location would depend on base sequence

One would picture that the RNA of the ribosome, which is a high-polymer RNA and presumably contains the information on specific sequence, would base-pair with the adaptor molecules Then, when two contiguous ones came together, the amino acids could react with each other to form the peptide bond.

The important thing about this idea was that it gave a highly specific way for the amino acid to locate itself on the template and could account for the high specificity of protein synthesis and failure to make mistakes.

Smithies. You could feed in energy, too, through the formation of the first bonds.

Hoagland: Yes The two important things about the soluble RNA are that it preserves the amino acid in activated form and it reacts *specifically* with amino acids apparently one RNA for each amino acid

One of the predictions of the adaptor hypothesis is that it should be necessary to have individual enzymes to attach individual amino acids to individual soluble RNA molecules. This does seem to be the case There wouldn't be any *a priori* reason for predicting that different amino acids would have to be activated by different enzymes until it became obvious that they had to be attached to different RNA molecules.

Smithies: At the level of amino acid activation, is there already any distinction between the different enzymes?

Hoagland: Yes Each enzyme is specific for a particular amino acid.

Lederberg And the same enzyme activates the amino acid and then transfers the amino acid to RNA?

Hoagland: Yes, that is what seems to occur The evidence is quite good for that now, because individual activating enzymes have been isolated free of RNA and have been shown to transfer only that amino acid which they activate to RNA. There is the exception of Berg's group (10) We don't have enough data now to know whether there are other exceptions and how this is going to fit together. But, in general, I think it is safe to say that the same enzyme does both jobs and that these enzymes are broadly distributed in living forms

It has been found in a number of laboratories that the soluble RNA participates in a special reaction which is necessary before it can accept amino acids. This involves the attachment of two CMP's and one AMP to the end of the RNA chain. The amino acid is then attached to this

terminal adenosine moiety as an ester on the ribose hydroxyl. If the end of the RNA is removed, the amino acid can no longer be attached

Anfinsen: Wouldn't this breed out whatever specificity there was at the end of the original RNA?

Hoagland: This certainly means that the code, the specific base sequence, is deeper in the molecule

If the adaptor hypothesis is correct, it might be predicted that those reactions up to the point where the template is involved would not be species-specific, *because all organisms deal with the same twenty amino* acids and sequence does not come in as a consideration until the template is reached With a few possible exceptions, in general it is true that activating enzymes and soluble RNA and ribosomes from different sources can be used interchangeably and the amino acids will be made into protein We shall hope that the protein made is that characteristic of the organism from which the *ribosomes* were derived!

Levinthal: In this picture of the activating enzymes and the soluble RNA, the information for the code is contained in the activating enzymes and therefore must be contained in the genes which control them We know that there are differences in DNA composition from species to species, which could reflect a difference in the code, if the genes controlling the activating enzymes had mutated The species independence of the soluble RNA and the activating enzymes has, to my knowledge, not been looked for in cases where there is a known difference in the DNA composition of the species

Hoagland That's right Also, though we can use these intermediates interchangeably, the sensitivity of our methods for detecting synthesized specific protein is not great enough to know whether or not we are just making junk protein at the end

Lederberg. Mammals and bacteria have been interchanged, haven't they?

Levinthal: But they happen to have been mammals and bacteria which have the same base composition

Hoagland: We have found that the soluble RNA will enter the ribosomes in company with an amino acid The question is, does it come out again after the amino acid is turned over to peptide linkage in protein? In other words, is there cycling, as the adaptor hypothesis suggests? We find that if particles with labeled soluble RNA are incubated with unlabeled soluble RNA, then labeled soluble RNA appears in the medium This suggests that cycling does occur

Smithies: Can an activated amino acid be transferred to a smaller portion of RNA, such as a tetranucleotide?

Hoagland: Many experiments have been done along these lines, degrading RNA by various means and attempting to put amino acids on fragments In general, this has been unsuccessful The molecular weight of this material seems to be of the order of 30,000 to 50,000, and any attempt to break it down to smaller subunits has failed. Furthermore, we have been unable to get any evidence that the unit consisting of the amino acid attached to the complete RNA molecule is broken down before it enters the ribosomes.

Smithies · Have you investigated the attachment of the amino acid to the RNA?

Hoagland · We have tried a number of ways of degrading the RNA enzymatically to oligonucleotide fragments, and, in the presence of CTP and ATP and the appropriate enzymes, we have tried to put the right end groups on these oligonucleotides to see if they would then accept amino acids So far, we have been unsuccessful We have also looked without success for acid-soluble nucleotide-amino acid intermediates during the course of amino acid transfer from RNA to protein

Lederberg: Isn't it a fact that there are smaller amino acyl adenylates that might be expected to be quite unstable, if they were of a small size?

Hoagland · We were looking for a step beyond the enzyme-bound adenylates, however

Lederberg · You can't even preclude that the larger adaptor transfers the amino acid in the ribosome to a triplet adaptor or something of that sort before it goes on to the template

Hoagland No, this cannot be excluded, either. The soluble RNA contains, interestingly enough, a number of odd bases, such as the pseudo-uridine that Cohn (16) and Davis and Allen (22) have described, and a number of rare methylated bases which are in higher amounts in soluble RNA than in other cellular RNA fractions What the meaning of this is, we don't know

Spiegelman. Dr Hoagland, have you looked at the base ratios of the SRNA from the mammalian systems?

Hoagland Yes

Spiegelman · Do they Watson-Crick?

Lederberg · Or Chargaff?

Hoagland: Are they complementary? Not particularly.

Spiegelman. The SRNA of *E coli* appears to complement quite well.

Hoagland: I should say that the base ratios in all the soluble RNA's from bacterial and mammalian systems look much alike and don't differ very strikingly from the particle RNA itself.

Spiegelman: Then there is an inconsistency of fact Paul Berg* has found that the base ratio of SRNA is quite different from that contained in the particles

Hoagland: I have not been impressed by the differences But with better analyses and better ratios, there may be a difference Nor was I impressed with the difference in Tissiere's data from our (40)†

Lederberg: I think one of the dilemmas of the doctrine is that nucleic acid recognizes nucleic acid, and possibly amino acid recognizes amino acid; an express procedure for bringing them together should be devised Is there any possibility that the enzyme has within it a segment of polynucleotide which is the basis of the recognition for its coupling to amino acid?

Hoagland: Apparently, activating enzymes can be purified free of nucleotide material

Lederberg: To the point where it is known that there is less than one triplet per enzyme molecule?

Hoagland: I think that can be eliminated

Spiegelman: It should be possible to find that It has been done with some labeled material

Lederberg: So there is a steric surface of protein which is able to distinguish one kind of polynucleotide from another?

Spiegelman: It may be distinguishing the amino acids

Lederberg: Yes.

*Personal communication

†EDITOR'S NOTE Dr Hoagland would like to add the following to his remarks at the Conference

The disagreement may be partly resolved by more recent data Soluble RNA from *E coli* does appear to have a high guanine and cytosine content and a low uracil and adenine content, which suggests complementarity This also seems to be true for yeast Rat liver SRNA is variously reported as having a high guanine and cytosine content, and only a high guanine content Ascites tumor SRNA has a high guanine but not a high cytosine content Were it not for the low cytosine content in these mammalian SRNA s the generalization could not be made that there is complementarity in SRNA with a considerable excess of guanine and cytosine over uracil and adenine What is quite striking, however is that although in mammalian tissues the base compositions of SRNA and particle RNA are very similar—both showing the high guanine and usually high cytosine content—this is not true for yeast and bacteria where all four bases seem to be more equally represented

The data thus far do not permit the conclusion that the SRNA is any more complementary than the particle RNA in either mammals or bacteria It does however point up the important fact that there may be more interspecies variation in base composition in particle RNA than in SRNA This is just what we would expect from our theory—SRNA dealing only with amino acid recognition, would be the same from species to species since all organisms use the same amino acids Ribosomal RNA, being the template, would reflect species differences More refined analyses may resolve the discrepancies (Most of the base analyses mentioned have not yet been published but are tabulated and discussed in *The Nucleic Acids* [40]).

Davis. There is nothing inherent in the RNA to permit a single strand to have the right coil, is there? I assume the helices must come at one peptide bond distance (Figure 22)

Hoagland: I don't think we even know what the form of the RNA in the particles is This was just a convenient way of writing it. I don't want to imply at all that it is helical. There is some evidence that it may be helical in particles, but this was just essentially a way of giving the idea that the adaptors would line themselves up, by complementary pairing, in such a way that the amino acid would be brought closer together Incidentally, this common CCA ending might be a way to bring the amino acid together, by having a reaching mechanism here, so that you could get closer But one could conceive of many ways in which this particle RNA would be arranged to permit this to occur. We don't know The difficulty is that we don't know what size piece actually goes into the particle.

Spiegelman: As the RNA comes out of the particle, does it still have the CCA terminal?

Hoagland: The CCA end accompanies the amino acid into the particle, and we don't know whether it comes out again but we would like to find that out.

Ingram: Dr Anfinsen, would you tell us something about the system with which you are studying the relationship between gene and protein structure?

Anfinsen: The enzyme with which progeny phage escape from host cells appears to be lysozyme (63) Whether or not lysozyme has anything to do with penetration of phage in the first place is not known, but it does seem to be concerned with the release of progeny

The amount of enzyme produced in an infected *E. coli* is many times more than is necessary for the production of complete phage particles within this cell, even if several molecules of lysozyme per phage are allowed By growing large cultures, up to 200 or 300 liters, we can isolate from the lysates of these cultures adequate amounts of lysozyme for chemical work.

The 300-liter batches are allowed to lyse, or if lysis is inhibited, are lysed artificially with chloroform We then make the usual low pH precipitation, during which all the cells, cell walls, and debris sediment to the bottom of the tank with the lysozyme attached, presumably, to the cell walls.

This debris can now be resuspended and treated with RNAase and DNAase, whereupon the lysozyme in the precipitate becomes liberated

We then add a large batch of XE-64 ion exchanger, to which the lysozyme adheres, being a very basic protein.

The enzyme is then eluted from the XE-64 and put on an XE-64 column, or more recently a carboxymethylcellulose column This small peak (Figure 23) emerges as the last component during gradient elution, and contains the lysozyme activity * The yields from one 300-liter fermenter are usually of the order of 100 to 200 mg protein . As far as we can tell, the material that is obtained by this purification is homogeneous If we examine it by the Archibald ultracentrifuge method, we find evidence for only one component. The enzyme has not been studied extensively in terms of its detailed chemical structure, although we do feel certain that it is a single chain and has, at the most, only one disulfide bridge, or possibly none I rather hope that it won't have any, because that means that the study of its function in relation to structure will be that much easier It will be a lot more like papain and easily subject to stepwise degradation with leucine amino pepti-

*Dreyer, W J, Crumpton, M, and Anfinsen, C B Unpublished results

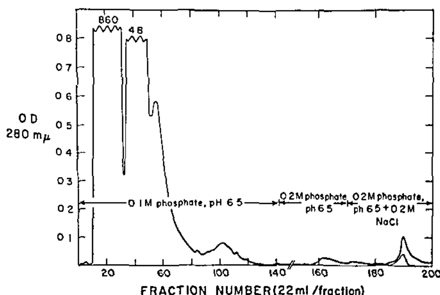


FIGURE 23. The purification of lysozyme from lysates of *E. coli* infected with bacteriophage T2 The small chromatographic peak at the far right contains the lysozyme activity (XE-64 cation exchanger) Reprinted, by permission, from Anfinsen, C. B *The Molecular Basis of Evolution* New York, John Wiley & Sons, 1959 (p 183)

the gels represent real protein components and are not caused by artifacts of the method, and I might mention some measures we have taken to establish this We can, for example, cut out one of the zones, isolate the protein from the gel, and store it at room temperature or in the cold room, and then put it back into the gel and observe its mobility No change, such as a reversion to multiple zones, occurs We can also obtain the same type of patterns in acid solution and in the presence of urea, so I think it fair to say that it is very probable that the zones which we observe in the gels do indeed represent distinct stable proteins

Spiegelman How do you prepare the material before you put it on the starch?

Smithies· Either whole serum or purified preparations will give the same results

Spiegelman· But, at the present moment, your evidence that the haptoglobins are distinct proteins is based essentially on their mobility?

Smithies. It is based chiefly on their behavior in starch gels and on the fact that the components can be isolated from the gels without changing their properties But, since we see the same sorts of differences between the various haptoglobin components over wide ranges of pH (from 2 to 9) and in the presence of urea at high concentration, I think we have very convincing evidence for the distinctness of the components. Ultracentrifugal studies of the purified haptoglobins confirm the results of the starch gel electrophoresis experiments

However, perhaps more striking evidence for the reality of the components is the observation that differences in the genetic constitution of an individual can cause the multiple haptoglobin zones to differ both in their relative proportions and in their relative mobilities

Figure 25 shows the starch gel electrophoretic behavior of all the types which we have had in sufficient quantity to purify (94) Type 1-1, in which a single molecular species of haptoglobin is found, occurs in whites with a frequency of about 15 per cent; type 2-2, with a whole series of haptoglobins, occurs with a frequency of something like 35 per cent, and the heterozygous type 2-1 occurs with a frequency of about 50 per cent The type 2-1 (modified) occurs frequently in Negro populations and is a heterozygous type very similar to that observed in whites, but, as is evident, the proportions of the multiple zones are different in the modified type 2-1 from those in the usual type 2-1. This difference is heritable and appears to result from the fact that the gene Hp^2 is, in these individuals, replaced by a slightly different gene, which can be designated as Hp^{2M} for the moment The Johnson type is a much less common genetic variant, in which the multiple haptoglobin zones

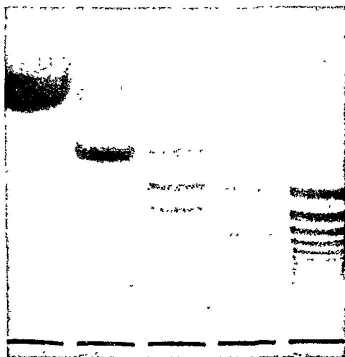


FIGURE 25 A comparison of purified haptoglobins of the types (left to right) 1-1, 2-1 (modified), 2-1, Johnson type, and 2-2 Electrophoresis was carried out in a starch gel, pH 8.5, made with a dilute borate buffer The proteins were stained with Amido-black 10B Reprinted, by permission, from Smithies, O, and Connell, G E Biochemical aspects of the inherited variations in human serum haptoglobins and transferrins In *Biochemistry of Human Genetics* G E W Wolstenholme, and C M O'Connor, Editors London, Churchill, 1959 (p 179)

differ from all the usual types both in their mobilities and in their relative proportions The pattern of inheritance of this type is not known, it has only been seen in two individuals—a mother and her daughter.

Ingram Have you seen the homozygote of the modified type 2-1?

Smithies: I haven't, but I think, perhaps, Dr Steinberg or Dr Sutton might know more about this, as they have studied populations in which they have reason to believe that they have observed homozygotes

Steinberg We have four families in which both parents are type 2-1 (modified) Among the seventeen children in these families, there was one child who was phenotypically type 2-2 On the basis of this one child, it looks as though the phenotype may, at least on occasion, be type 2-2 when the Hp^{2M} allele is homozygous (32).

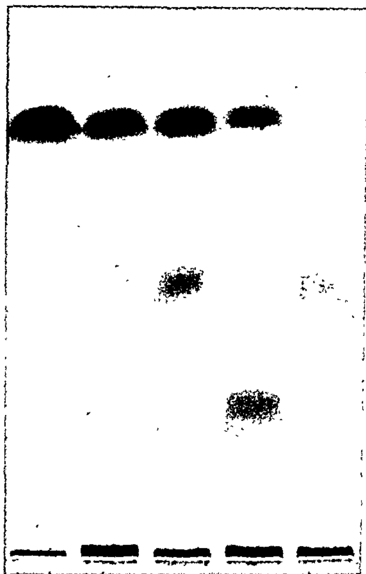


FIGURE 26. A comparison of the five types of haptoglobin (illustrated in Figure 25) after treatment with mercaptoethanol and iodoacetamide (95). The samples were of the types (left to right) 1-1, 2-1 (modified), 2-1, Johnson type, and 2-2. Electrophoresis was carried out in a starch gel, pH 3 2, made with a dilute formic acid/sodium hydroxide buffer

Smithies: The differences observed in these several haptoglobin types are rather remarkable, and yet, at least in the case of the three major types, there is very little doubt that they are caused by changes in the genes at a single locus.

Dr Connell and I, therefore, tried the effect on the haptoglobins (purified from serum by the use of ion-exchange resins [17]) of a variety of chemical reagents in the hope of recognizing more clearly the products of gene action in this system. Reductive cleavage proved to be the most useful procedure (94) and we now use mercaptoethanol as the reductant, followed by iodoacetamide to protect the -SH groups The cleavage can be carried out in the presence or absence of urea, although it is a much more rapid reaction in the presence of urea which suggests that the relevant -SS- bonds are to some extent inaccessible For example, at pH 9 in the presence of 8 M urea and 0 02 M mercaptoethanol, the reaction takes place in less time than we can measure, but in the absence of urea it takes something like 4 to 5 hours even with 25 times the concentration of mercaptoethanol

We had difficulty in studying the products of the reductive cleavage in alkaline gel systems, but recently we have been using acidic gels, and many of our difficulties have been resolved Figure 26 shows the behavior in a formic acid/sodium hydroxide gel (pH around 3.2) of the five haptoglobin types (illustrated in Figure 25) after they have been treated with mercaptoethanol and iodoacetamide in the presence of urea It is evident that some of the cleavage products do not migrate in the formic acid/sodium hydroxide gels, but that in all five types a portion of the haptoglobin molecule has been split off which does migrate, and which still differs in the several types

The result of these procedures is that we can now detect in each of the several genetic forms of haptoglobin a portion of the molecule which is recognizably related to the corresponding genotype Thus, in each of the two homozygous types (1-1 and 2-2), a single migrating component is observed, the one related to the gene Hp^1 differing from that related to the gene Hp^2 In the heterozygous type (2-1), both gene products, if I may call them that, are present The other heterozygous type (2-1, modified) shows one product identical with that related to the gene Hp^1, but the other product is not identical with, although it is similar to, that related to the gene Hp^2 This agrees with the limited information available on the inheritance of this type, which is compatible with the suggestion that it corresponds to a genotype Hp^{2M}/Hp^1. The Johnson type gives two migrating products and is therefore probably a heterozygous type, but both of these products differ from those

related to the genes Hp^1 and Hp^2 Possibly this type, which was observed in a Negro woman and her daughter (by Dr. E. R. Giblett, King County Central Blood Bank, Seattle, Wash), may be the result of a rare dominant modifier gene at some locus other than the *Hp* locus.

Spiegelman· Are the genes unlinked?

Smithies Family studies indicate that the three common types and the type 2-1 (modified) are caused by the action of allelic genes, but there is insufficient information on the inheritance of the Johnson type to be able to say anything about linkage in the case of this type

The differences in the shapes and mobilities in the starch gels of the zones corresponding to the gene products are very helpful in that they suggest that the product related to the gene Hp^1 is very slightly aggregated in the formic acid/sodium hydroxide gel system, and that the products related to the genes Hp^2 and Hp^{2M} are very considerably aggregated One of the products in the Johnson type appears to be essentially free from aggregation (the faster migrating zone), while the other is even more aggregated than the Hp^2 gene product This is of particular interest since the multiple zones in the untreated haptoglobins are observed only in the types in which the genes Hp^2 and Hp^{2M} are present, and in the Johnson type

Wagner. Do these proteins stain with benzidine?

Smithies· No they do not. The gels I have illustrated are all stained with a protein stain

Spiegelman. I wanted to ask, was there any significance to the fact that there was quite a bit more material left behind in two of those?

Smithies. I am inclined to think not All the protein will enter the gel if the concentration of urea is increased sufficiently; this brings me to a consideration of the effect of urea on the electrophoretic behavior of these cleavage products

In the presence of 2 M urea (and formic acid/sodium hydroxide buffer), the aggregation of the products related to the genes Hp^1, Hp^2, and Hp^{2M} is abolished, but not until the urea concentration is increased to around 4 M does the slower of the products from the Johnson type cease to aggregate However, at all concentrations of urea, even at 8 M, the mobilities of the products related to the genes Hp^1, Hp^2, Hp^{2M}, and both of those obtained from the Johnson type, are all recognizably different from each other, so that it seems as though we can now obtain a small portion of the haptoglobin molecule which carries with it the genetic information which distinguishes the several types. I should add that in the presence of 8 M urea the material left at the origin of the gels (in the absence of urea) migrates, and that all the types behave

similarly with regard to this part of the molecule, although we cannot be quite certain yet that this portion is identical in all types However, I do not think that this will prevent us from determining the nature of the gene action in the haptoglobins by studying the structure of the smaller fragments which you have seen migrate in acid gels in the absence of urea

In the near future, we hope to isolate these migrating products and to do "fingerprints" on them, in order to establish the difference in the amino acid composition of the several haptoglobin gene products We must also find out how these smaller portions of the molecule are combined with the remainder of the molecule, which may be common to all the types, in such a way that in most types we observe multiple components and in one we observe only a single molecular species of haptoglobin

To summarize, we have been able to break down the haptoglobins in such a way that we can obtain products recognizably related to the genotypes of the persons from whom the haptoglobins were isolated

Motulsky: What method do you use for isolation?

Smithies: We use a method involving ion exchange (17) The serum is adjusted to a pH of about 4 2, at which pH most of the serum proteins are positively charged, and to the serum is then added the positively charged resin Dowex-2 This takes out the haptoglobins and leaves most of the other serum proteins in solution Elution is carried out so as to emphasize this selection still further, and high purity haptoglobins are obtained in one step

Recently Dr G E Connell and Mr R W Shaw, both of the Department of Biochemistry, University of Toronto, have scaled up the preparation by introducing DEAE cellulose as the ion-exchange medium, and they have carried out further purifications on the eluate This is particularly important for structural studies in which very highly purified material must be used

Motulsky: And was the electromobility of the isolated products performed with starch grain or with filter paper electrophoresis?

Smithies: Unfortunately, the purified haptoglobins are strongly adsorbed to filter paper and do not migrate I do not know how they behave during electrophoresis in starch grains. When whole serum is used, a small difference in filter-paper mobility can be seen in the haptoglobins of the three common types When they are complexed with hemoglobin, this difference is much greater and is readily demonstrable by any of the conventional electrophoretic methods

Lederberg: Have you or Dr Ingram examined mouse hemoglobin, which forms a series that reminds me a little of this?

Smithies: I did not know that mouse hemoglobin showed series similar to the haptoglobins.

Lederberg: There is a polymorphism there.

Ingram Yes, there is, but one of the types has multiple hemoglobin bands

Smithies: I do not think that multiple bands of this type are particularly rare They are observed, for example, in some cases of multiple myeloma where apparently abnormal serum proteins are formed My present feeling is that the multiple components in the haptoglobins represent stable polymers, but the situation is not one of simple polymerization Possible polymers of albumin of a similar type have turned up too in some clinical cases (79)

Itano: In some cases, multiple components may be association products Svedberg and Hedenius reported that the hemoglobin of some reptiles and amphibia contains rapidly sedimenting components which they thought corresponded to dimers and trimers of the hemoglobin molecule (98)

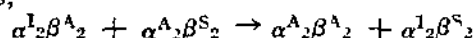
Neel: In mouse hemoglobin, one gene substitution seems associated with the clear appearance of two additional bands, but this involves us in the question of minor components of hemoglobin.

Anfinsen It may be dangerous to say much about these things, even in your system, Dr Smithies To the best of my knowledge, there has been no clear-cut chemical evidence that explains this kind of microheterogeneity Most cases are explainable on the basis of either polymerization or interaction with other proteins or with small uncharged molecules For example, with sheep ribonuclease, eight components which have shown no obvious chemical differences are obtained Genetic determination is not imperative in such situations if the interaction hypothesis is followed. You may be observing a very localized change in the secondary structure of a particular protein which permits it to exhibit an unusual physical property.

Ingram: Dr Itano has done some interesting studies of hemoglobin dissociation, which I should like to discuss now.

Itano. Hemoglobin A is composed of a pair of α-chains and a pair of β-chains, so that the molecule can be formulated as $\alpha^{A}{}_{2}\beta^{A}{}_{2}$. Hemoglobins S and C, which have abnormal β-chains, are $\alpha^{A}{}_{2}\beta^{S}{}_{2}$ and $\alpha^{A}{}_{2}\beta^{C}{}_{2}$, respectively Since each of these molecules is symmetrical, it seems reasonable to assume that genetic determination of the structure of half-molecules, $\alpha^{A}\beta^{A}$, $\alpha^{A}\beta^{S}$, and $\alpha^{A}\beta^{C}$, respectively, followed by the association of the half-molecules would be sufficient to account for the structure of hemoglobin This assumption seems to be contradicted by

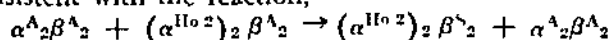
the fact that heterozygous cells do not contain hybrid molecules such as $\alpha^{A}{}_{2}\beta^{A}\beta^{S}$, which would result from the association of two different half-molecules. Hemoglobins A, S, and C dissociate into subunits in acid and reassociate in neutral solution (26,54). Attempts to produce hybrid molecules that contain two different β-chains by dissociating and re-associating mixtures of these hemoglobins were unsuccessful, and further investigation showed that acid dissociation does not yield half-molecules $\alpha\beta$ but yields subunits α_2 and β_2. Exchange of these subunits in recombined mixtures was demonstrated with use of labeled hemoglobin molecules (91). The inherited alteration of hemoglobin I is in the α-chain, and recombination of hemoglobin I with hemoglobin S yielded hemoglobin A and a hemoglobin composed of the abnormal α-chain of I and the abnormal β-chain of S (51). The reaction is represented as,



$$\alpha^{I}{}_{2}\beta^{A}{}_{2} + \alpha^{A}{}_{2}\beta^{S}{}_{2} \rightarrow \alpha^{A}{}_{2}\beta^{A}{}_{2} + \alpha^{I}{}_{2}\beta^{S}{}_{2}$$

Recombination of hemoglobin I with hemoglobin C resulted in increase in the proportion of an electrophoretic component with the mobility of hemoglobin A but without the appearance of a new component. It was postulated that the increased positive charge of the β-chain of hemoglobin C was neutralized by an increased negative charge of equal magnitude in the α-chain of hemoglobin I, so that a molecule composed of a pair of each of these chains has the same mobility as hemoglobin A.

We have applied this technique to hemoglobin Ho-2. According to Smith and Torbert, the genes for hemoglobins S and Ho-2 segregate independently (92), and double heterozygotes contain S, Ho-2, and A in their erythrocytes. Our recombination experiments indicate that the defect of Ho-2 resides in the α-chain (52,53,55). The fact that the electrophoretic abnormalities of S and Ho-2 are opposite and approximately equal suggested to us that, as in the recombination of hemoglobins I and C, a doubly abnormal molecule with the mobility of hemoglobin A is present. Recently, we were able to effect a partial chromatographic separation of the intermediate electrophoretic component, the one with the mobility of hemoglobin A. Acidification and neutralization of this component resulted in net formation of S and Ho-2 (53), a result consistent with the reaction,



$$\alpha^{A}{}_{2}\beta^{A}{}_{2} + (\alpha^{Ho\text{-}2})_{2}\,\beta^{A}{}_{2} \rightarrow (\alpha^{Ho\text{-}2})_{2}\,\beta^{S}{}_{2} + \alpha^{A}{}_{2}\beta^{A}{}_{2}$$

Double heterozygotes therefore have four hemoglobins, one of which contains two inherited abnormalities and has the electrophoretic and chromatographic behavior of hemoglobin A. With respect to these criteria for separation, the doubly abnormal molecule is a hidden mutant product.

Lederberg. What were the relative amounts of the components?

Itano: According to Smith and Torbert, the proportions of S, A, and Ho-2, as determined by filter paper electrophoresis, are about 45, 45, and 10 per cent, respectively. The proportion of A appears to be too low. In sickle-cell trait, the usual proportion of S is 30 to 40 per cent In Ho-2 trait, the proportion of Ho-2 is 15 to 20 per cent (92) In other words, Ho-2 is made much more slowly than A, and S is made somewhat more slowly than A Therefore, the proportion of the doubly abnormal molecule in double heterozygotes would be expected to be less than the proportion of A.

Neel. In this particular family, what was the proportion of hemoglobin S in the plain sickle-cell trait individuals?

Itano· This has not been determined

Neel What about plain Ho-2 heterozygotes?

Itano. Fifteen to 20 per cent.

Neel Are you disturbed by the proportions of hemoglobins S, A, and Ho-2? On a quick calculation, these proportions raise the question of whether there is random association of the types of molecules I just set up a checkerboard, with 35 per cent hemoglobin S and 65 per cent A on one margin and 20 and 80 per cent on the other. The expected proportions on random association would be 59 per cent of hemoglobin "A," when it is assumed that the double abnormality moves with the A, 28 per cent hemoglobin S, and 13 per cent Ho-2. The actual results are different from what would be expected on a random basis I'm trying to determine whether or not there is any assortative pairing between the alphas and betas

Itano· The proportions of hemoglobins S, A, and Ho-2 in one of our moving boundary experiments were 40 per cent, 49 per cent, and 11 per cent, respectively These figures are not corrected for boundary anomalies in the dilute buffer used In general, we have found that the apparent proportion of the most rapid component, in this case hemoglobin S, is higher than the true proportion under our conditions of electrophoresis

Stern· Would such a calculation really be expected to be right? Is it not true that from one individual to the other, if they are simply heterozygotes, there is a varying percentage of hemoglobins? The factors which determine differences in the speed of synthesis in different individuals are of an unknown nature. Therefore, is it permissible to compare the proportions of one or two such double heterozygotes with the proportions of single heterozygotes, which are also very few in number, as in the case of Ho-2?

Neel: It is true that there are modifying factors There are also significant intrafamily similarities in the proportion of abnormal hemoglobin in the simple S trait No, this is not a calculation where the details would be insisted upon, but it is a general guide to what is occurring It is worth pursuing For instance, the figures Dr Itano gives now are close enough so that it looks all right

Stern: Are these latter figures on a different individual from the one you mentioned before?

Neel: They are on the same person but different methods were used to obtain them.

Smithies I would be surprised to find that the proportions of the different association pairs were randomly determined

Neel: I don't know whether I'm surprised or not, but it does introduce another order into this process of protein synthesis

Lederberg: Does this family provide evidence for the selective association of products of linked genes? I assume the loci are only distantly linked if they are linked at all But if both abnormalities are transmitted from the same parent, would there be a higher proportion of doubly abnormal molecules than if the double heterozygous individual received the traits from different parents? Is there an example of the latter mating?

Itano: No, there is no example of a double heterozygote individual who has received the traits from different parents.

Lederberg. What is the recombination value between the two genes in that pedigree?

Steinberg: There are eight out of ten

Neel: You can't make any case for linkage

Lederberg: In the first generation, one out of seven, and three out of ten.

Glass: One out of five in the first The next generation goes up

Lederberg: You said that the fact that you get out four kinds of tetramers is evidence of the independent formation of the dimers Isn't it just possible that, instead, there are four kinds of ribosomes in which the two pieces are arranged in a different way? Isn't it possible that they are, in fact, made together, the alpha and beta being made on a given ribosome, with four kinds of ribosomes?

Ingram: Yes, I suppose it is conceivable I think the kind of double mutant that Dr. Itano showed should be clearly distinguished from the hidden mutations we talked about earlier Here, there are two defects which compensate electrophoretically, whereas the hidden mutations

usually referred to are likely to be single mutational events, by and large

Neel. I'll accept Dr Itano's double mutant as a hidden mutation In the G/S family, there is another problem. This is the presence of thalassemia, which affects the synthesis of hemoglobin A in a manner which is not at all clearly understood. Consequently, there can be another reason for the absence of "A" in the two AG individuals But that still doesn't take away the evidence for genetic segregation between the G and the S loci.

Lederberg How certain is it that the hemoglobin A in thalassemia is, in fact, hemoglobin A?

Ingram: It's not at all certain, but this is a point under investigation at the moment. We have discussed the genetic and chemical constitution of adult hemoglobins, and I would next like to mention very briefly fetal hemoglobin (hemoglobin F), which is chemically distinct from adult hemoglobin by a variety of tests In particular, it is much more resistant to alkaline denaturation, and it has a different absorption spectrum in the UV

Fremont-Smith: How early does fetal hemoglobin appear?

Ingram Some say there is a form that comes before the fetal hemoglobin, it is called primitive hemoglobin Others think this is an artifact of the experimenter's methods So the story is confused at the moment

Kretchmer: Has the question of primitive hemoglobin versus hemoglobin H actually been investigated thoroughly?

Ingram: No It is, of course, particularly difficult to obtain this material

Fremont-Smith: There would be no difficulty at all in Sweden and Finland, where legal abortions make available for study a great deal of living human fetal material

Neel Japan would be even better because there are more people.

Ingram: Fetal hemoglobin is roughly the same size and shape as adult hemoglobin, and it is made up of four polypeptide chains, as is adult hemoglobin My former colleague, Dr Hunt, has recently shown that hemoglobin F contains two α-chains which are like the adult α-chains by fingerprinting (44). They are the same in gross structure certainly, and possibly also in every detail. This is not yet absolutely certain, but I think it can be assumed for the time being that they are the same chains.

The other two chains of fetal hemoglobin, however, are different We call them γ-chains to denote the fact that, although they are the

same length as β-chains, they are chemically different Their fingerprints are more different than the fingerprints of hemoglobins A and S, for example, but there are also points of similarity in the peptide structure.

We would like to postulate that because fetal hemoglobin is composed of the same α-chains as adult hemoglobin, in fetal hemoglobin the same alpha genes control the formation of α-chains A separate set of genes controls the formation of γ-chains This gives us three sets of genes—the α-chain genes, the β-chain genes, and the γ-chain genes in the developing erythrocytes

If the development of the embryo is traced all the way through to the adult, it could be said that the α-chain genes are always active, but that in the embryo the γ-chain genes are active and the β-chain genes are silent Then, there is a gradual transition to active β-chain genes, with the corresponding fall in activity of the γ-chain genes This argument is by no means watertight, and it depends on the assumption that fetal hemoglobin and adult hemoglobin occur together in the same erythrocyte, however, they might be distributed amongst different cell populations Dr Itano showed quite some time ago that in cases of homozygous sickle-cell anemia, all the cells can be made to sickle, indicating that all the cells contain hemoglobin S, and suggesting, in turn, that they all contain hemoglobin F This occurrence of fetal hemoglobin in an adult, in the case of these hemoglobinopathies, is quite characteristic. Rich (80) first suggested that fetal hemoglobin in these abnormalities is produced as a compensating mechanism

Fremont-Smith: What about the fetal hemoglobin in a homozygous hemoglobin S fetus?

Ingram: It is normal fetal hemoglobin

Neel: If the hemoglobin S change is in the β-chain and fetal hemoglobin does not have that chain, it should be normal

Lederberg· I don't see why it is necessary for your argument that hemoglobins F and A be produced in the same cells There could be a system of differentiation involving two kinds of cells

Ingram: It is not absolutely necessary to the argument, but it seems likely that they are produced in the same cells

In cases where one or both of the β-chain genes are abnormal and something like hemoglobin S is produced, it can be assumed that this abnormal hemoglobin S is produced at a much reduced rate compared to normal hemoglobin It looks as though, in compensation, the γ-chain genes are activated with the production of fetal hemoglobin Mutations in the α-chain gene are very much rarer. In such a case, there would be no compensating production of fetal hemoglobin But I don't

know of any case of a homozygote carrying an α-chain mutant This is where the absence of compensation would be found. It is possible that an α-chain mutation, with consequent reduction in the rate of α-chains, is a much more lethal situation because it affects the production of fetal hemoglobin also and makes the fetus less viable

Itano. In most cases of thalassemia-C disease, there is a preferential reduction of hemoglobin A However, one family has been reported (107) in which both hemoglobins A and C were reduced in absolute amounts in an individual who was presumed to have inherited both the C and thalassemia genes This effect would be consistent with a defect in the production of α-chains since the same α-chain is present in both hemoglobins A and C

Anfinsen· Is there anything to rule out the possibility that another set of symbols really should be tried? Perhaps the normal beta gene and fetal gamma gene are really the same, and perhaps synthesis of the β-chain of hemoglobin S is, for that matter, also controlled by the same gene Chemical differences might reflect only intracistronic modifications rather than the influence of entirely different genes

Ingram: If this were assumed, it would be necessary to discard all the ideas about coding and template hypothesis, because there is a whole set of new amino acids present

Anfinsen· No, they aren't that different

Ingram I disagree, because there are some fundamental differences For example, adult hemoglobin contains absolutely no isoleucine as a very striking characteristic of its composition, whereas the γ-chain of fetal hemoglobin does The terminal amino acid of the β-chains of adult and γ-chains of fetal hemoglobin are quite different, in that the latter has glycine instead of valine, and the tryptophan content is entirely different

Neel· Earlier in the discussion, something was made of the fact that there are no known homozygotes for mutations involving the α-chain It is worth pointing out that the three mutations localized to this chain, Ho-2, I, and D_α, are all rare, and homozygotes are very unlikely on purely statistical grounds

Fremont-Smith: Is it significant that the fetal hemoglobin is produced in a different part of the body? Is it not true that fetal hemoglobin is not produced in bone marrow?

Kretchmer There are many more hematopoietic systems for producing blood cells or erythrocytes in the fetus than there are in the adult individual There is a strong possibility that certain cells which contain fetal hemoglobin only may be produced by fetal hematopoietic centers,

and when the child is born, there is a reduction of these centers and new centers now produce cells which contain all adult hemoglobin

Ingram: Cells which contain only hemoglobin F and no S would not sickle, therefore, since all the cells sickle, they must all contain hemoglobin S

Kretchmer. Every one of them sickles?

Itano: I have not compared percentage of sickling cells and percentage of fetal hemoglobin myself; however, I have cited instances in the literature in which samples with 100 per cent sickling have been reported to have fetal hemoglobin

Neel: It was done by Karl Singer (90)

Motulsky: Betke (11) in Germany has developed a staining technique which preferentially washes out adult hemoglobin from erythrocytes but it does not wash out fetal hemoglobin He takes blood films from newborn or young infants and, with this stain, shows some cells *that are completely blank and therefore contain only adult hemoglobin* Other cells are fully stained and therefore have only fetal hemoglobin In addition, he could demonstrate all transitions from blank to full staining, indicating that many erythrocytes contain both fetal and adult hemoglobin

Fremont-Smith: Both in the same cell?

Motulsky: Yes There is also some indirect evidence in myeloid metaplasia in adults where the bone marrow may be almost completely inactive and the patients' blood formation occurs in fetal centers such as spleen and liver You would think they might have large amounts of fetal hemoglobin but they don't

Fremont-Smith: In connection with the Betke (11) staining method, it should be possible to make studies of newborn or infant bone marrow to determine whether or not fetal hemoglobin is being produced at all in the bone marrow It seems to me worthwhile to think of the organ systems involved as well as the chemistry

Cotterman: Dr Kretchmer's postulate, that there might be, at one stage, some cells that are making adult hemoglobin and others that are making fetal hemoglobin, represents a kind of mosaic situation, presumably not on the basis of any genetic difference but as a developmental process I am particularly interested in genetic mosaics and especially with reference to blood cell antigens In this case, there is the possibility of demonstrating genetic differences in individual erythrocytes. It might be worthwhile mentioning one way in which the blood antigens might be useful to those in the hemoglobin field, clarifying some of the questions that have been raised here

Unlike the case in erythrocyte antigens (18), the techniques being used in hemoglobin research are not nearly sensitive enough to enable detection of hemoglobin differences in individual cells I find on questioning several here that they too feel that this is not, perhaps, an exaggeration However, it might be possible to achieve the same end result by techniques that do not involve measurements on single cells If there were some properties of cells with their different hemoglobins that would allow them first to be separated, it would be possible to take a mixture of such cells, separate them on this basis, lyse them, and assay the hemoglobin solutions by the usual techniques

The properties that suggest themselves as a means of separating cells are, of course, the blood groups themselves This would suppose that there were linkage of some blood group gene with one of the hemoglobin genes By making separations first on a serological basis, we would, in some instances, be performing a separation that is nearly complete with respect to the hemoglobin factors We might solve the problem in this way

Marks Is there any evidence for linkage between a blood group gene and a hemoglobin gene?

Cotterman I believe not, at present Snyder and associates (96) once reported a linkage for sickling and MN types, but Neel and Hanig found negative results *

Neel We have been unable to show linkage between hemoglobin S and some of the blood groups (76) Also, ovalocytosis is not associated, as far as we know, with the hemoglobin abnormalities.

Lederberg If adult hemoglobin has no isoleucine and fetal hemoglobin does, I suggest there really should be no difficulty whatever in microbiological assay of the isoleucine in a single erythrocyte

Ingram It won't be quite clean, because there is a small percentage of other proteins in the cell, and the isoleucine content of fetal hemoglobin is small

*EDITOR'S NOTE Dr Cotterman would like to add the following "afterthought" to his remarks at the Conference

Application of the method described above need not be delayed until family studies establish a suitable case of linkage The demonstration of erythrocyte mosaicism with respect to hemoglobins, following separation for any one of the many known blood group antigens, could, in itself, furnish a means of detecting linkage, for example, the blood of a Negro heterozygous for the Kell antigen (K/k) as well as the sickle-cell gene (A/S) If a small proportion of cells was found to be negative for K (inagglutinable with anti-K) and also lacking or markedly deficient in hemoglobin S, the result would be difficult to explain except in terms of linkage Loss of a chromosome or chromosome segment carrying both S and K, and crossing-over in the same region, are two kinds of somatic "mutational" events that could account for an association of this kind

Lederberg: I feel that it should be possible to do an assay of the amino acid content of the erythrocyte to an accuracy of 10 per cent by microbiological methods.

Steinberg: Lewis antigen is not present in the newborn and does not appear until about one year of age I wonder if this fact could be used as a method of separating cells I don't know whether or not anyone has related this to the type of cells It isn't on the cells

Atwood: It would go onto the cells if they were put in the right plasma, but I don't think it would distinguish one kind of cell from another.

Steinberg: However, as the child ages, it does go on the cell

Lederberg: Because it is produced in the serum

Steinberg: Wouldn't this help to separate it?

Atwood: More likely, it would go equally onto any cell, whether it has fetal or other hemoglobins I think single-cell methods are more promising than blood group methods

Itano: Other evidence for the presence of both hemoglobins S and F in the same cells can be found in some work by Schneider (84), who studied infants with sickle-cell trait The proportions of hemoglobins A, S, and F and the percentage of sickling cells were determined In some cases, the percentage of hemoglobin F plus the percentage of sickling exceeded 100

Lederberg: Is there any possibility of cataphoresis of intact erythrocytes to display the content of hemoglobin?

Ingram: No, their movement in an electrical field is determined by their outer surface and particularly by the ions which are absorbed

Davis: Have any experiments been made to determine what factors induce the shift from fetal toward adult, such as oxygen tension, CO_2 tension, etc ?

Ingram: No Many people are becoming very interested in this, and it is, of course, a very important problem But, to my knowledge, there have been no data, not even any hypotheses

Fremont-Smith One possibility might be to study premature infants, because they are exposed to the new oxygen environment 2 or 3 weeks earlier.

Ingram: It's fairly clear that oxygen isn't the only factor involved.

Fremont-Smith: The rate is no different in the development of the fetal hemoglobin in the premature infant?

Ingram: No The curve that I drew earlier of the fall in the production of fetal hemoglobin and the rise in the production of adult hemoglobin does not show any discontinuity at birth

Fremont-Smith: But there is no difference in the premature infant? It would be interesting to get the fall and rise in the premature infant.

Kretchmer: The falloff curve has been done. It starts higher and it comes down faster But it is not much faster in the premature infant.

Ingram: The important thing is that there is no discontinuity.

Lederberg Is there no particular increase of hemoglobin F after adaptation to high altitudes?

Motulsky: No This has been investigated.

Ingram: I have already mentioned the existence of normal minor components of hemoglobin One of these is hemoglobin A_2 (64), which is shown in Figure 27. This is a photograph by Dr. Park Gerald of a starch-block electrophoresis of normal hemoglobin, with the major A component preceded by a "snout" similar to the one which Dr Itano described in boundary electrophoresis H. G Kunkel calls this snout A_3, but it may be hemoglobin from old cells. Migrating more slowly is hemoglobin A_2, which is present to the extent of about 2 5 per cent in normal individuals It is electrophoretically quite distinct from hemoglobin A_1

There are probably about another half dozen normal minor components at low concentrations, adding up to a total of about 10 per cent These are being purified by W A. Schroeder and his colleagues by chromatographic procedures. The chemistry of these minor components is being worked out, but until it is known, we can't say anything about how they got there A few of the apparent minor components might be artifacts of preparation

However, hemoglobin A_2 has been studied in considerable detail. On the bottom line of Figure 27 is the blood of an AS heterozygote, showing hemoglobins A and S as major components. Hemoglobin A_2 is still present in its normal position, with nothing behind the A_2 position It has been the experience of everyone that A_2 is unaffected by the presence of abnormal hemoglobins such as S, C, and E These are hemoglobins in which the abnormalities are on the β-chain, suggesting that hemoglobin A_2 does not contain a β-chain under the control of the usual β^A gene. In the case of hemoglobin C heterozygotes, the hemoglobin A_2 is not clearly visible, because it has a very similar electrophoretic mobility

The chemical studies being carried out now, both in our laboratory by Mr. A O W Stretton, and also at California Institute of Technology, show that hemoglobin A_2 is very similar to hemoglobin A, but that there is at least one peptide difference, and there may be more The peptide difference is in peptide No. 26, the β-chain peptide altered in hemoglobin

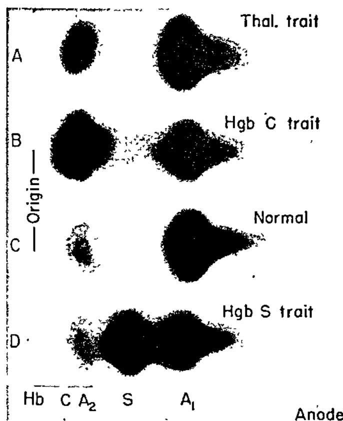


FIGURE 27 Starch block analysis of various bloods illustrating the minor hemoglobin component A_2 (Photograph by courtesy of Dr Park S Gerald)

E Of course, this is tantamount to saying that hemoglobin A_2 contains a β-chain, and I have just said that β-chain mutations, such as in hemoglobin S, do not affect A_2 So we must adduce that hemoglobin A_2 contains peptide chains which are chemically like the β-chains but which are under separate genetic control

It appears that hemoglobin A_2 probably contains normal α-chains Thus, it seems that the α-chains run through the whole series of hemoglobins from the adult hemoglobins to the fetal hemoglobins to this minor component of hemoglobin

We believe, for the time being, that there is another type of chain involved in the rest of the molecule, which, for the present, we would like to call δ^{A_2} There may be many points of chemical similarity

between δ-chains and β-chains, but they seem to be under separate genetic control. We can write the genotype of hemoglobin A_2 as

$$\alpha^A / \alpha^A, \delta^{A_2} / \delta^{A_2},$$

with the same alpha gene operative for all three types of hemoglobins—a rather extraordinary situation. This may very well have some evolutionary significance, but what it is, I don't think we can discuss at the moment

Thalassemia is an inherited disease which is characterized by a microcytic anemia. It can exist both as a carrier trait, thalassemia minor, or as apparent homozygote, thalassemia major. It can exist as a third form of the disease called intermediate thalassemia, of intermediate severity In thalassemia minor or thalassemia trait, there is apparently normal hemoglobin A It moves electrophoretically like hemoglobin A, and answers to all the gross chemical tests as does hemoglobin A. But the amounts present within the cell in the typical thalassemia trait seem to be reduced

A comparison (Figure 27) of the intensity of the hemoglobin A_2 spot in thalassemia trait with the intensity for normal blood will show that there is a doubling of the amount of hemoglobin A_2 in this example of thalassemia trait We do not know why this should occur Whether it is a compensating mechanism, although a rather feeble one, we don't know, either

In thalassemia major, with certain conditions, there can be complete or almost complete absence of hemoglobin A. Of course, these subjects are very ill, and in compensation there is production of fetal hemoglobin once again as in the S/S homozygote This is one kind of thalassemia; however, many different kinds of thalassemia occur throughout the world.

Wagner. What happens to hemoglobin A_2 in thalassemia major?

Motulsky: It is normal in relation to the total hemoglobin, both fetal and adult It is increased in relation to the adult hemoglobin fraction.

Lederberg: Did you say there is no hemoglobin A, or it is greatly reduced?

Ingram: It is greatly reduced, and there are a few cases known where there is no hemoglobin A.

Fremont-Smith: At the moment, in thalassemia, you don't have any absolute changes in hemoglobin A_2 in any of the conditions? Is that correct to say?

Ingram: No, the thalassemia minor traits fall into two categories: those that have an increased A_2, and they are, perhaps, the more numerous ones, and those that do not have an increased A_2. But within each

class, there is a great variability in expression of the thalassemia gene, which has led many to suppose that there is not one thalassemia gene but many.

Marks: What was the concentration of hemoglobin A_2 in the subject with hemoglobin I?

Ingram: I would expect it not to be elevated and, if anything, reduced, in view of what I am going to say next

There is another characteristic of some types of thalassemia When they occur together with the hemoglobin S trait to give an S/thalassemia double heterozygote, a condition results which is clinically as severe as sickle-cell anemia in most cases Such an individual, in a typical case, has hemoglobin S and some fetal hemoglobin but virtually no hemoglobin A, at least they have very little This is called an interacting thalassemia-sickle-cell double heterozygote It looks as though, in most cases, the thalassemia gene suppresses the formation of normal hemoglobin specifically without affecting the production of hemoglobin S This is true for several other combinations of abnormal hemoglobins and thalassemias, such as hemoglobin C-thalassemia, hemoglobin D-thalassemia, and hemoglobin E-thalassemia

If thalassemia specifically represses the formation of hemoglobin A, this rules out a number of theories of what thalassemia might be For example, if it is postulated that thalassemia is genetic interference with one of the many enzyme systems involved in heme synthesis, leading to a microcytic anemia, it is difficult to see how reduction in total heme available to the synthetic mechanism would specifically reduce the production of normal hemoglobin and not affect the production of hemoglobin S at the same time The same holds for more generalized concepts of suppressor genes, and there also it is difficult at the moment to see how they could reduce specifically hemoglobin A manufacture But we must also say that there are so many different kinds of thalassemia known that it is quite likely that certain cases of thalassemia are simply suppressors or are interfering with heme production, or are interfering with some more general phenomenon connected with hemoglobin synthesis The majority probably do not fall into that class

The hypothesis I advance, which was worked out by Mr Stretton and me (48), and which we call the substitution hypothesis, is as follows We do not claim any very great originality for this idea, as Dr Itano mentioned the same sort of idea (49), and in that article, he quoted Linus Pauling as putting forward somewhat similar ideas The hypothesis is simply this There is no essential difference between a thalassemia mutation and other abnormal hemoglobin mutations such as S or C,

except that a thalassemia mutation is an amino acid substitution in either the α-chain or the β-chain which leads to *no electrophoretic change*

A consequence of this hypothesis is the specificity which is necessary to explain the S-thalassemia double heterozygote, which produces nothing but hemoglobin S with high fetal hemoglobin as a compensating mechanism Such a double heterozygote would make perfectly normal α-chains, but one β-chain gene would be β^S and the other β-chain gene would be β^{th}. The β^{th} gene would have the property that the production of abnormal β-chains is greatly reduced and may be blocked altogether It is easy to see how one might get a wide range of possible effects, depending on what kind of amino acid substitution is involved.

It is known that in the electrophoretic substitutions where there is a recognizable abnormal hemoglobin, there are hemoglobins which are grossly abnormal in function. On the other hand, there are hemoglobins which, although electrophoretically different, are biologically normal And there are probably all sorts in between. In the case of the non-electrophoretic substitutions, there should also be a whole spectrum of different types of abnormal β-chains, all the way from the ones which are so grossly nonfunctional that the β-chain is not made at all, as in interacting S/thalassemia, to those where the β-chain is only mildly abnormal and where it is synthesized to a certain extent.

The same is true of a plain thalassemia minor. If it is assumed that a particular β-chain mutation is severe in terms of producing abnormal hemoglobin, a reduction in the total amount of hemoglobin made might be expected, which is what is found But there is no interference with δ-chain formation, which is concerned with hemoglobin A_2, and hemoglobin A_2 manufacture is therefore unaffected by this type of mutation

A β-thalassemia mutation, β^{th_1}, coming together with a different β-thalassemia mutation, β^{th_2}, in a double heterozygote, might very well have the appearance of what is normally described as a thalassemia homozygote Here, there is interference with β-chain production on a grand scale, and maybe it is so severe that no hemoglobin A is made at all. On the other hand, an α-chain thalassemia, α^{th_1}, would not necessarily interact in a sickle-cell thalassemia double heterozygote α^{th}/α^A, β^S/β^A. Such cases are known in which the thalassemia, although present, does not lead to a complete reduction of hemoglobin A synthesis and does not lead to such a severe clinical picture. In such a case, it is still possible to make hemoglobin A from this combination of genes.

This, then, is a possible explanation of the *noninteracting* type of thalassemia.

If an α-chain thalassemia and a β-chain thalassemia come together, there might be a partial reduction in hemoglobin A synthesis, depending on the severity of the abnormality in each case By such a scheme, a very broad spectrum of abnormalities is possible Another interesting consequence of the α-chain thalassemia is that interference with hemoglobin F synthesis might be expected, since that also contains α-chains Similarly, interference with hemoglobin A_2 production might be expected This is a possible explanation of the fact that in some thalassemia minors there is not an increased level of A_2 Perhaps these are α-chain mutations which do not allow the increase in production of hemoglobin A_2

In contradiction to this picture, hemoglobin H, another inherited abnormality of hemoglobin, is peculiar in its instability, and it has been shown to be composed entirely of β-chains (57) The β-chains of this β_4 aggregate are interchangeable with the β-chains of normal hemoglobin, so they are the same in gross structure, but they may not be the same in detail.

We would like to speculate that such a case results from two α-chain thalassemias, leading to an excess in production of β-chains which then associate Another possibility is that it results from a combination of an α-chain thalassemia with a particular β-chain mutation, which we can call β^H, such that there is also a nonelectrophoretic mutation in the β-chain produced by this gene According to our definition, it would be a thalassemia, but involving an abnormality of the β-chain which has the specific effect of increasing the likelihood of association to β_4 I don't think too much of this picture, but I think it is something that should be considered

Sutton· What is the hemoglobin A_2 status?

Motulsky: The hemoglobin A_2 is normal or low in hemoglobin H patients as well as in their thalassemic relatives

Ingram· It would agree but it would not distinguish between either of those two hypotheses

Neel: Isn't there a third possibility? I believe that in most of these hemoglobin A_2 families, only one of the parents has thalassemia. Isn't that right?

Motulsky Yes.

Neel· Therefore, why couldn't it be an α^{II} thalassemia?

Ingram· That is essentially the first hypothesis, except that you're

calling it something else You're using a different symbol. This would be an α-chain thalassemia together with α^H

Motulsky: In your system, if you call it α^H, you might say the parent is normal by all criteria, but may still carry this mutation a hidden mutation which becomes phenotypically apparent in the presence of thalassemia.

Ingram: One of the parents does have thalassemia.

Neel: This is a terminological problem, but thalassemia is associated in the minds of people with a cytological effect.

Ingram Yes, but just because the other parent doesn't show thalassemia doesn't mean to say that he or she doesn't have a mild defect of this kind When the two come together, they produce the grossest abnormality of hemoglobin A.

Sutton. Have the β-chains been fingerprinted?

Ingram: Yes

Sutton: Is there any defect?

Ingram: No, but then it would not be expected because it is non-electrophoretic, and any changes would hardly show up

Lederberg. I have a rather fanciful alternative to suggest, but there is a precedent for it in *Salmonella* There might be a locus or an allele at the α-locus which is making a gene product which is indistinguishable from the typical β-chains In the heterozygotes, α^A/α^H, β^A/β^A, there would be half the production of normal α-chains with a 1 to 3 ratio of alphas to betas In the homozygote, if there is one, there would be nothing but β-chains. If there were an α thalassemia combined with H, α^{th}/α^H, then there should be reduced α^{th}-chains and the rest would all be β-chains

Ingram: Yes, but you would have to transfer a great many differences.

Lederberg: It might very well involve a duplication or a bodily transfer of that gene We found exactly that in a *Salmonella* stock, which should have been diphasic, producing two alternate types of flagella, but instead, in this particular stock at the H-1 locus, we had a gene which was making a typical H-2 product That is the only reason I would dare suggest it

If it were just a consequence of decreased production of α-chains, I would expect β_4 to be produced in many of the other cases where there is a great diminution in the production of α-chain. Apparently, that is not enough to cause aggregation of β-chains.

Ingram: Hemoglobin H is becoming more frequent every year. It is characterized by physical instability of the product with such things as

inclusion bodies formed in the cell There may be physical reasons why it is not frequently found.

Motulsky: I am convinced that many cases of hemoglobin H have been overlooked. Small quantities of hemoglobin H easily precipitate out, and unless special care is taken when examining fresh specimens, they may not be detected

Ingram: The interesting thing to me is that no one has yet found a hemoglobin which is α_4. But this is presumably a matter of time

Motulsky. The pedigrees usually show one parent with thalassemia and without hemoglobin H and the other parent normal Children in these sibships can have thalassemia without hemoglobin H, hemoglobin H only, or normal hemoglobin

Neel: Do some children have both hemoglobin H and thalassemia?

Motulsky: Yes, presumably

Ingram: There was a somewhat analogous situation in an abnormal fetal hemoglobin, which I have already mentioned J A Hunt has shown Bart's hemoglobin to have a chemical constitution of δ^F_4 This is an analogous product to the β_4 of hemoglobin H The presence of thalassemia is suspected, but these are newborn children and the diagnosis of thalassemia is very tricky

Smithies: It is certainly odd that we never see any of this β_4 or α_4 in the normal individual, if it can be formed

Sutton: Has the formation of tetramers in a normal person been properly excluded?

Motulsky: You don't see it electrophoretically in the small quantities expected

Sutton: But you perhaps would not if it were there in only a half per cent or less.

Smithies: What are we assuming with respect to RNA in the heterozygous individuals? Are there two separate RNA's? Perhaps this would have a bearing on whether or not a single alpha gene would be expected to produce its normal amount of α-chain regardless of what the other alpha gene does I would say that without any compensating mechanisms, we should never, on this basis, have any reason to assume that there would be less than half the amount of normal α-chain in an α^A/α^{th} case Do you see many cases in which the normal α-chain is less than half?

Itano: In terms of the average content of an intact cell, there is evidence for compensation For example, in homozygous C, the mean corpuscular hemoglobin might be somewhere around 24 micromicrograms, of which 12 is normal α-chain In the normal person, the mean

corpuscular hemoglobin is something like 30 micromicrograms, of which 15 is normal α-chain

Lederberg. That is exactly the point The ratio between the α- and the β-chains remains constant

Ingram: Within a cell, yes

Lederberg: And it is proportional to the number and consecutiveness of the α- and β-chains that you have given here While you can expand the total rate of production of both chains, they have to expand together. It's hard to see how this could happen if different ribosomes are involved in making the two products In a single ribosome, both alpha and beta information could get to the heme, so the heme can act as the common inducer for both of them to come off

Stern: Couldn't the abnormal chain clog up both kinds of ribosomes?

Ingram Yes, it could, presumably But let me get back to the original hypothesis and point out that it is possible to test it If there is anything in the idea that the thalassemias are nonelectrophoretic amino acid substitutions, we should be able to verify it in cases of hemoglobin A which we have reason to believe have been affected by thalassemia.

There is a complete alternative to the substitution hypothesis I call it the "tap" hypothesis This uses as its model the Freese model of gene structure (27) On this basis, there would be a hemoglobin gene connected by a "link" to the rest of the genes This could perfectly well be DNA of protein but it doesn't matter for our purposes. Within the developing erythrocyte, very few genes are active, most of them are silent If we make the further assumption that the "link" is the tap which turns on the particular gene which follows it, one tap would turn on the α-chain gene and somewhere else another tap would turn on the β-chain gene Thalassemia might then be a mutation which affects the tap, which determines the degree to which the gene is activated, and which determines the rate at which the α-chains are produced

On such a simple picture, all the genetics which we have just discussed will be indistinguishable, particularly if it is assumed, as Freese does, that an adjacent region of this DNA structure determines the structure or the activity of the black box But there is a very important difference When this gene is turned on at all, it will make a perfectly normal type of α-chain, there would be no amino acid substitution, but the yield would vary depending on what thalassemia has done to the black box.

Lederberg: If you want to pursue your previous discussion, you must say that the tap for the alpha and the tap for the beta are coupled You must maintain a constant ratio of production.

Ingram: Physical equilibrium can explain the constant ratio It

could be coupled, but it need not be I think Dr. Itano's experiments indicated that it is not coupled, because he obtained the independent assortment of four possible products.

Itano. But some sort of control of relative amounts produced in a cell must exist, since the sum of the mass of α-chains equals that of the β-chains whether a cell is normocytic or microcytic and whether or not a cell is heterozygous Hemoglobin H appears to be an exception to this rule

Lederberg: I can't help but remark that this is precisely the model for which we have just a little more evidence for *Salmonella* phase variation The additional evidence is that there it is possible to obtain recurrent events which turn the tap on and off without affecting at all the antigenic specificity of the product, which is in the immediate neighborhood.

Ingram. It would be necessary in thalassemia to speculate that the degree to which the tap is opened varies

Lederberg: I wouldn't want to say that we have a sensitive test for quantitative variation in this expression

Levinthal: In a sense, all that can be said here is that differentiation does take place, of which we are all aware Markert (68), for example, shows that different proteins do come and go during the course of development

Lederberg: This is not a trivial point The burden of most genetical theories of development is that variations occur in the cytoplasm but that there is really no important change in the chromosome

Atwood: Do you prefer an all-or-none tap? It would work You just have to say that it turns on later and stays on for shorter times, and there is less production

Ingram: All right

Marks What type of mechanism would you say determines whether the tap is on or off?

Ingram: I think it was Freese who added the further elaboration that the black box is determined by the DNA which immediately precedes it. At any rate, it is the genetic constitution of this adjacent region which determines the tap and which, in turn, determines whether or not the gene is able to function

Lederberg What determines the state of the tap?

Ingram: We don't know, but the problem of fetal and adult hemoglobin leads directly into a very good experimental approach

Motulsky: Our thinking about thalassemia has arrived at the same conclusions as Dr Ingram's on somewhat different grounds. We are

impressed with the increasing genetic evidence that the kind of thalassemia seen in Southern Europeans, associated with elevated hemoglobin A_2 levels in heterozygotes, is allelic to the hemoglobin β-chain locus Since the thalassemia depresses hemoglobin synthesis (or in these cases β-polypeptide synthesis specifically), a homozygote for the thalassemia, allelic to the hemoglobin beta locus, would be very deficient in β-chains However, α-chain production is not affected in these patients and the "dormant" γ-chain gene compensates to produce γ-chains again, so that homozygotes with this type of thalassemia have large amounts of fetal hemoglobin ($\alpha^{+}{}_2\,\gamma^{+}{}_2$) (Figure 28)

Figure 29 shows the interaction of this thalassemia gene with the hemoglobin S gene The single dose of the thalassemia gene leads to marked suppression of synthesis of normal $\beta^{+}{}_2$-chains. Since the other β gene carries the sickling mutation and only makes $\beta^{S}{}_2$-chains, the high ratio of S hemoglobin ($\alpha^{+}{}_2\,\beta^{S}{}_2$) is explained.

Figure 30 shows the type of thalassemia seen in hemoglobin H patients Such patients inherit a thalassemia gene from one parent. Presumably, this kind of thalassemia depresses α-chain formation and is not associated with hemoglobin A_2 elevation. The α-chain inherited from the other, phenotypically normal parent may be normal ($\alpha^{+}{}_2$), as shown in Figure 30, or may be phenotypically silent but with a hidden mutation ($\alpha^{H}{}_2$) (69) The deficiency of normal α-chains would lead to excess β-chain formation, as a result of which the β-chains join up with each other to form β_4 molecules or hemoglobin H

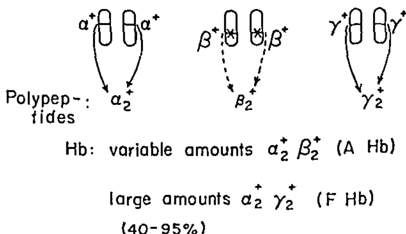
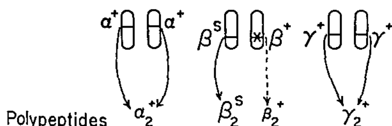


FIGURE 28 Suggested genotype of individuals with thalassemia major (high A_2).



Hb: large amounts $\alpha_2^+ \beta_2^S$ (S Hb) (60–90 %)
small amounts $\alpha_2^+ \beta_2^+$ (A Hb)
small amounts $\alpha_2^+ \gamma_2^+$ (F Hb)

FIGURE 29 Suggested genotype of individual heterozygous for both thalassemia and sickle cell hemoglobin (high A_2)

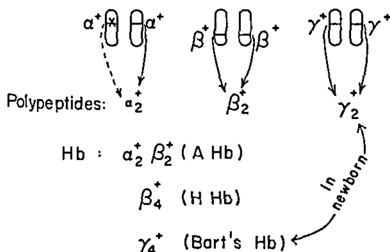


FIGURE 30 Suggested genotype for individuals with thalassemia major in families in which hemoglobin H is found

Thalassemia with α-chain depression should lead to $\gamma^{+}{}_{4}$ or Bart's hemoglobin in the newborn or infant, since during this age period γ-chain production rather than β-chain production is active. Bart's hemoglobin actually has been found. In some patients, mixtures of $\beta^{+}{}_{4}$ (Hb H) and $\gamma^{+}{}_{4}$ (Bart's hemoglobin) have been detected

Figure 31 depicts the formation of the three types (α, β, and γ) of hemoglobin chains during early life. The curve is a composite of many data on the amount of fetal hemoglobin before and after birth Fetal hemoglobin, or more accurately the γ-chain, only persists in trace amounts after 4 to 6 months of age Since the α-chain is present in both adult and fetal hemoglobin, it presumably is produced both before and after birth These curves explain why diminished amounts of hemoglobin S or hemoglobin C—both β-chain mutations—are found at birth Recent instances of fetal hemoglobin variants (other than Bart's hemoglobin) which disappear after a few months of life most likely will turn out to be γ-chain mutations. α-Chain mutations should show up at birth, no case has been reported yet.

As mentioned earlier, a number of cases have been found in Negroes

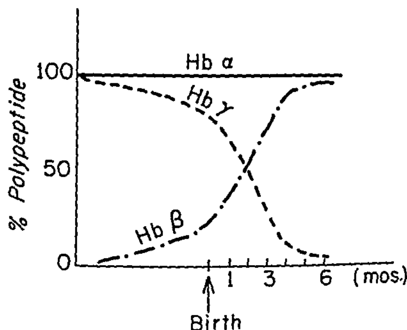


FIGURE 31 The relative production of β-chains and γ-chains before birth and in the neonatal period (α-chain production has arbitrarily been set at 100 per cent).

where large amounts (10 to 35 per cent) of fetal hemoglobin persist in adults unassociated with other diseases A focus for this dominant trait appears to be in Liberia. The condition has been variably called hereditary persistence of fetal hemoglobin, nonmicrocythemic thalassemia, or high hemoglobin F gene We have studied a large family where this gene is present in different family members either with A, S, or C hemoglobin, respectively When present with hemoglobin S or C, no hemoglobin A could be demonstrated, as is frequently seen in the interacting kind of thalassemia. These findings strongly suggest that the basic defect leading to high fetal hemoglobin in adults cannot be the hereditary persistence of the γ-chain Under such a hypothesis, a patient carrying both the high F and the sickling genes would still have a single fully functioning β^+ gene making normal $\beta^{+}{}_2$-chains, which should form normal A hemoglobin $(\alpha^{+}{}_2\ \beta^{+}{}_2)$ Actually, no hemoglobin A could be demonstrated. It is therefore more likely that the basic defect in these patients is similar to that seen in patients with the β-chain suppressing thalassemia as shown in Figure 28

Another point in favor of this interpretation is suggestive genetic evidence that in such families the gene producing the condition is allelic to the hemoglobin β-chain locus Figure 32 shows a mating of a sickle-cell trait mother with a father who had 70 per cent C and 30 per cent F hemoglobin (this man's sister, not shown on the pedigree, had 70 per cent A and 30 per cent F) Among five offspring, three had hemoglobins C and S, one had hemoglobins C and A, and one was a double heterozygote for sickling and the high F gene The genetic interpretation with allelism is shown on Figure 33 Under the alternate genetic interpretation of inheritance on different chromosomes, shown in Figure 34, eight different types of offspring would be expected As will be noted, four

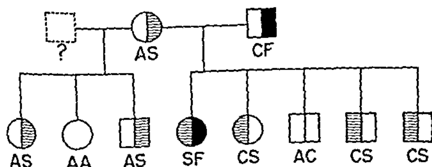


FIGURE 32 Pedigree of mating of sickle trait carrier with double heterozygote for hemoglobin C and high fetal gene

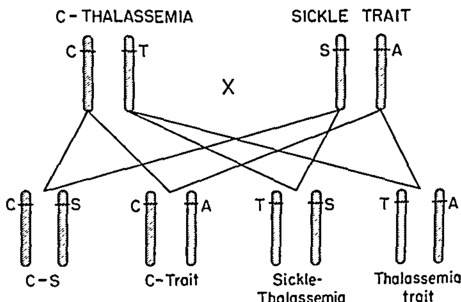


FIGURE 33 Genetics of a mating such as shown in Figure 32, if high fetal gene and hemoglobin S (or C) gene were allelic (only the β-chain genes are diagrammed) The word thalassemia or the symbol T have been used rather than the term "high F"

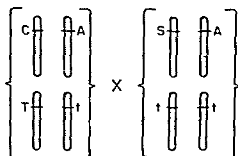
types are identical under both schemes while four different types (sickle-cell trait, normal, C and S with high F, and C with high F) would be expected under the hypothesis of inheritance on different chromosomes In the family under study, none of these four types was found This is significant for the allelic scheme at about a 5 per cent level. It is not water-tight, and more critical matings are needed

Fremont-Smith: I would like to ask a question about the "dogma" Which of these two alternative hypotheses, if either, is necessitated by the dogma· Can the gene operate only in one highly specified environment and therefore perform only one function? Would any other environment either suppress its activity or be lethal? Or can a gene perform a variety of functions, depending upon the environment to which it is exposed? Is it normally exposed to a very limited environment? Is that why it behaves as consistently as it is supposed to do?

Lederberg: They are not mutually exclusive statements. But the gene either operates or does not operate. There is no qualitative difference in the product, depending on the environment.

Wagner: But that which the gene forms acts differently in different environments.

C-THALASSEMIA SICKLE TRAIT



EXPECTED OFFSPRING:

$\frac{1}{8}$ CS tt	$\frac{1}{8}$ CA tt	$\frac{1}{8}$ AS tt	$\frac{1}{8}$ AA tt
(CS)	(C-Trait)	(Sickle trait)	(Normal)
$\frac{1}{8}$ CS Tt	$\frac{1}{8}$ CA Tt	$\frac{1}{8}$ AS Tt	$\frac{1}{8}$ AA Tt
(C-S-Thal)	(C-Thal)	(Sickle-Thal.)	(Thal. trait)

FIGURE 34 Genetics of a mating such as shown in Figure 32 if high fetal gene and hemoglobin S (or C) genes were on different chromosomes The word thalassemia and the symbol T have been used rather than the term "high F"

Fremont-Smith It has no multiple potentiality at all?

Lederberg *Pleiotropism non est.*

Fremont-Smith. Did you add, at the "dogma' level?

Lederberg In terms of the primary product, that is the doctrine

Stern: As an example which fits this very well, there is a striking gene in *Drosophila* which performs three very different functions: It changes the nature of the wings, it changes the configuration of the thorax, and it adds a special organ, an extra sex comb, to the front legs There are no hormonal interactions between these three parts So it must be assumed that the gene in these different parts produces effects independently You might now say, does the gene do it by performing three primarily different functions or only one, which then later interacts with different cellular environments?

An answer is suggested by the finding of genetic inhibitors of *engrailed*, which is the name of this gene. These inhibitors affect all three developmentally quite independent effects (43) It would seem best to assume that the same primary product is inhibited in the three different organs

Spuhler: In man, hemoglobin S changes the shape of the skull, too It also fits the "dogma."

Stein Isn't that a secondary effect?

Spuhler We call it a pleiotropic effect.

Neel That is a "pedigree-of-causes" type of effect rather than a true pleiotropism

Lederberg: The dogma says "no." There are facts which are ambiguous.

Glass One of the most striking examples in the human species is the Pelger anomaly (71,72), where the shape of the nuclei in the granulocytes is changed in the heterozygous condition In the homozygous condition, there is a very striking series of effects on the development of the skeleton, which seems to have nothing at all to do with the shape of the nuclei in leukocytes But I think all of us are convinced that when we know what the primary effect of that gene is, we will find it to be according to the "dogma."

Lederberg Of course, in a way, this is re-defining what you mean by primary

Glass: Yes, it is

Lederberg For example, the α-chains of three different molecules, hemoglobin A, hemoglobin F, and hemoglobin A_2, are identical. In principle, they change at the alpha locus, so we now re-define primary to ask what the common effect is which lies behind all of these three changes. The most hopeful way of attacking the nucleic doctrine is to look for primary pleiotropism But, whenever this is done, it almost always reduces to this next problem.

Fremont-Smith: The gene is pushed back one step further.

Lederberg Not the gene, but the primary product You define as primary the one effect of the gene which can then explain all the manifold consequences But, so far, it has been possible to do this with respect to a protein. It has not been necessary to say that two different polypeptide sequences are determined by the same gene. However, there are many cases that have not yet been analyzed at this level, and there are a number of anomalies which I said were ambiguous. I don't believe there are any current facts to contradict this interpretation of the doctrine. If there were, we might even abandon it. There are ambiguous

facts and there are situations which were once ambiguous but which have now been resolved, either along the lines that Dr Stern has mentioned or by a more thoroughgoing morphogenetic analysis

Fremont-Smith: How do you deal with differentiation, on that basis?

Lederberg: If one adheres firmly to the nucleic doctrine, one might say that differentiation does not involve a change in nucleic information But this tells very little about where events in differentiation are taking place, because they could still be either on the chromosomes or in the cytoplasm, or both There is little concrete information on this point, and it might not be profitable to say very much more, except that there is at least one case where it has been possible to implicate the chromosomes in an event in differentiation This is the transplantation experiment of King and Briggs (62) One is then very much tempted to try to find other features of the chromosomes, not the nucleic acid sequence, which might be responsible At the moment this is pure hypothesis, although it gives a direction in which to work.

I gave this doctrine a name I wonder if it is acceptable I called it the nucleic doctrine

Davis: I think that is a euphemism The term *dogma*, has been used up to now.

SUMMARY

Bearn It is obviously very difficult, if not impossible, to summarize this discussion adequately. Also, I am somewhat inhibited by my attention having recently been drawn to a remark in *The Decline and Fall of the Roman Empire* When discussing one particular phase of history, Gibbon said, "A cloud of critics, compilers and commentators darkened the face of learning" Consequently, I shall merely recall the highlights of the discussion that has taken place here in the hope that they will provide a focus for further discussion and clarification

Dr *Ingram introduced the subject of the abnormal hemoglobins,* their chemistry and their genetics, and we learned that the abnormal hemoglobins have reached the letter R, leaving only eight more degrees of freedom before we reach the end of the alphabet The suggestion, made originally by Dr Neel, that we name abnormal hemoglobins by the region from which they were first discovered, has much to recommend it. Variants occur in both adult and fetal hemoglobin, the hemoglobin "Alexandria" being an example of the latter.

Chemically, it seems clear that hemoglobin is composed of four polypeptides consisting of two pairs of identical chains The α-chain begins with the sequence valine-leucine and the β-chain valine-histidine-leucine.

Recent findings indicate that these chains, isolated by countercurrent distribution, contain equal amounts of proline and are of special interest from a structural viewpoint, since the formation of an alpha helix is not possible at the site of a proline residue and some other folding of the molecule at these sites must occur

The postulate around which much of this discussion has revolved was whether the primary amino acid structure of the peptide chain by itself is sufficient to determine the secondary and tertiary structure of the final protein molecule This has been known colloquially throughout this Conference as the "dogma." Experiments involving denaturation of proteins lend some support to this hypothesis. Myoglobin can be denatured and then reconstituted and the x-ray crystallographers cannot, within certain limits, tell the difference. Whether the small differences observed are biological or technical is not yet clear

A discussion of sickle-cell anemia produced the comment by Dr. Neel that a handful of individuals homozygous for hemoglobin S maintain hemoglobin levels of about 11 gm. per cent and do not show any symptoms normally associated with hemoglobin S. In these rare cases, it must be presumed that the S gene interacts with another gene (which in some instances may be the high fetal gene) to give rise to an entirely different effect. However, these rare observations do not negate the widely held belief that most of the clinical picture can be laid directly at the door of the abnormal hemoglobin, although how the hyposthenuria can be caused by the sickle hemoglobins is admittedly far from clear

Fingerprint analysis of the partial tryptic digests, combined with methods designed to separate the α- and β-chains, have enabled the location of the amino acid substitution in the abnormal hemoglobins Thus. Hemoglobins S, C, and E, are substitutions on the β-chain, hemoglobins I and Hopkins-2 are α-chain lesions Fetal hemoglobin contains two γ-chains and hemoglobin H four β-chains Hemoglobin D is of particular interest, *since all three samples* of hemoglobin D examined differ and the mutations have affected either the α- or β-chains H. Lehmann has estimated that there are a million people in India affected by hemoglobin D, and it will be of great interest to determine the number of different mutations which have led to the formation of an abnormal hemoglobin which electrophoretically would be characterized as hemoglobin D The molecular nomenclature of the abnormal hemoglobins is a vexing subject, yet of considerable importance if those outside the field are not to be utterly confused. In general, it was agreed that hemoglobin A should be written $\alpha^{A}{}_2\beta^{A}{}_2$, hemoglobin S should be written

$\alpha^{A}{}_2\beta^{S}{}_2$, and hemoglobin I, $\alpha^{I}{}_2\beta^{A}{}_2$. Fetal hemoglobin should be $\alpha^{A}{}_2\gamma^{F}{}_2$. Dr. Motulsky leaned a little toward using $^{+}$ for the wild type. In his nomenclature, hemoglobin A would be $\alpha^{+}{}_2\beta^{+}{}_2$.

Hemoglobin G has raised problems for the "dogma" so frequently referred to during this conference. One family has been studied in great detail. One of the crucial points is that a man with hemoglobin G and S and thalassemia is married to a normal woman who has had a child who has neither hemoglobin G nor S but who does have thalassemia. Thus, the two genes S and G cannot be allelic, but Dr. Ingram tells us that these two nonallelic mutations have given rise to amino acid substitution in two neighboring amino acids. This is clearly anti-dogma and the family, which is currently under examination, remains puzzling. This family illustrates the importance of single pedigrees in which critical matings can reveal basic facts of the utmost importance.*

Although human hemoglobin is in many ways a very convenient protein for structural studies, it suffers from the limitations that it cannot be used for fine-structure genetic analysis. Dr. Levinthal's work on the alkaline phosphatase of *E. coli* is particularly suitable for fine-structure genetical analysis and was discussed next. In this system, it is relatively easy to pick up phosphatase-negative mutants, by irradiating the cells and examining 10,000 of them. One disadvantage of using bacterial enzymes for structural studies is that the amount of protein synthesized is small compared with the amount of hemoglobin and, moreover, the enzyme does not come neatly packaged within an erythrocyte. Nevertheless, if the organism is grown under conditions of phosphate limitation, the enzyme can account for perhaps 7 per cent of the total cell protein. This figure seems to be a fairly general one, if either full induction or complete release of repression can be attained.

Purified alkaline phosphatase has a molecular weight of 80,000 and is composed of two identical subunits. Use of an ingenious system in which the amount of DNA between two marker mutants and the amount of DNA between two phosphatase mutants is calculated seems to indicate that the size of the "gene" is of the order of 2000 nucleotide pairs. Since there are about 400 amino acids in a half-unit of alkaline phosphatase, this shows that approximately five nucleotide pairs (3 to 7) are responsible for the insertion of a single amino acid into the molecule. This ratio of DNA to protein (coding ratio) is much smaller than previously found. Experiments with tobacco mosaic virus gave a minimal coding ratio (in this case single-strand RNA) of about 50 to 1.

A discussion of the coding mechanism was introduced by Dr. Levin-

*EDITOR'S NOTE: See Dr. Neel's footnote on page 93.

thal. Since DNA contains only four bases and since there are twenty amino acids which must be coded, it is clear that a three-letter commaless code of the type introduced by F H. C. Crick is still the most satisfactory because it represents the minimum number of nucleotides which can convey the necessary information Mutation in a nucleotide sequence can give rise either to nonsense or mis-sense. The former would probably result in total absence of the protein whereas mis-sense would result in an altered protein. Depending on where in the protein molecule mis-sense occurred, the mutation might be important or not, while nonsense mutations would presumably always be serious. Theoretically, these mutations would be in the ratio of 1 to 1, and it is an interesting experimental fact that, at least in bacterial systems, half the mutants observed make a cross-reacting material and half do not. However, it was clear from the discussion that the question of the details of the code were still far from settled. One of the difficulties in applying this to the abnormal hemoglobins is that one can never know whether or not the mutation is one-step and, as Dr. Lederberg pointed out, we don't have any evidence that hemoglobin A is the ancestral hemoglobin in man However, a test of the validity of the commaless code might be possible by the use of selective chemicals at low concentration which should give rise to one-step mutations.

An interesting discussion centered around the possibility of detecting hidden (nonelectrophoretic) mutations in hemoglobin A. It seems possible to calculate very roughly that there have been a million cell division cycles, more or less, in the history of the human race. Another way of looking at this is to say that the probability of a mutation having occurred at any given human locus is about 1. Thus, barring selection, the probability of finding an amino acid substitution in a hemoglobin molecule should be high. However, since we know selection has been going on, these calculations must not be taken too seriously. Thus far no nonelectrophoretic mutations have been proved in man.*

Dr Hoagland filled the gap between DNA coding and the final synthesis of the protein molecule by introducing a discussion of the role of RNA and protein synthesis. Before protein synthesis can occur, each amino acid is activated by its own specific enzyme to form the aminoacyl adenylate. Then these amino acids become bound to soluble RNA (transfer RNA) and finally are transferred to particulate RNA in the ribosomes Before the discovery of transfer RNA, Crick had developed

*This subject was discussed later in the Conference (see page 149) by Dr Ingram in relation to his amino acid substitution hypothesis for thalassemia, in which he postulates that thalassemia may represent a hidden mutation of hemoglobin A

an adaptor hypothesis in which he postulated that amino acids might attach to a triplet of nucleotides which would be complementary (this is the important fact) to the triplet in the DNA strand It would be anticipated that activating enzymes and soluble RNA from different sources would still permit attachment of amino acids, and the evidence suggests that this occurs. Transfer RNA has a molecular weight of between 30 to 50 thousand It contains the unusual base pseudouridine as well as certain uncommon methylated bases At present, it is not possible to say whether these observations have biological significance.

Lysozyme is synthesized in large amounts in the cells of *E coli* infected with bacteriophage. The excess lysozyme is released into the medium following lysis If large cultures (200 to 300 liters) of *E. coli* are grown, lysozyme can be isolated in sufficient quantities to perform structural studies (100 to 200 mg) Dr Anfinsen has used this approach to study the relationship of genetic structure to protein structure Lysozyme, a strongly basic protein, is adsorbed to a polycarboxylic resin (XE-64) Following elution and chromatography on carboxymethylcellulose, lysozyme is obtained in a remarkably purified state. The enzyme, which appears to have a molecular weight between 13,500 and 14,500, consists of a single chain with probably only one disulfide bridge, although perhaps none exists A number of heat-stable and heat-labile mutants have been isolated but thus far no differences in the fingerprints of the T4 parent and the heat-stable mutant offspring have been detected However, if the differences in structure of the mutants are nonelectrophoretic, the fingerprint techniques would be less sensitive than if the substitution affected the over-all charge of the molecule

Dr Smithies continued the discussion with an account of the haptoglobin system in man There are three main haptoglobin types in man which are described as types 1-1, 2-1, and 2-2. In the starch gel system, type 1-1 has a single band and types 2-1 and 2-2 multiple bands. One of the most interesting features of the heterozygote (type 2-1) is that protein bands exist which are electrophoretically and ultracentrifugally different from those seen in either homozygote A haptoglobin variant exists in Negro populations in which the relative proportions of the type 2-1 bands differ. Other rare variants of the classical haptoglobin types have been observed and further examples undoubtedly will be disclosed in the next few years Structural studies in which the chromatographically purified haptoglobins have been treated with urea and mercaptoethanol before electrophoresis in starch gel reveal single bands in the homozygotes (types 1-1 and 2-2) and a mixture of the two in

the heterozygote. These proteins are presumably related to the primary gene products The modified type 2-1 results from a normal Hp^1 gene but the Hp^2 gene is abnormal. Fingerprint analysis on the various purified haptoglobins will be awaited with considerable interest.

Wagner. The discussion was continued by Dr. Itano with a consideration of reassortment of the components of hemoglobin molecules Each hemoglobin molecule is made up of 2 α- and 2 β-chains. One might expect α_4 and β_4 or other combinations to be made in addition to the normal $\alpha_2\beta_2$ molecule, or mixtures of S and A β-chains in individuals heterozygous for S However, such hybrids do not seem to occur ordinarily

It is known that treatment of native hemoglobin with acid does cause the molecule to dissociate, but only into α_2 and β_2 components and not into $\alpha\beta$ fragments When the solution is neutralized, the original $\alpha_2\beta_2$ type molecule is reconstituted. Hence, if one causes the dissociation of hemoglobin S, with the constitution $\alpha^A{}_2\beta^S{}_2$, in the presence of hemoglobin A, with the constitution $\alpha^A{}_2\beta^A{}_2$, no hybrid molecule should be expected after subsequent reassociation However, by labeling the two halves, it is possible to show that reassortment between the α_2 and β_2 halves does occur after dissociation *in vitro*. In addition, combinations of hemoglobin I with C and S should give hybrids such as $\alpha^I{}_2\beta^S{}_2$, since I is an α-chain mutation and C and S β-chain mutations This was in fact confirmed by experiment, but again *in vitro*, since individuals doubly heterozygous for I and C or S are not known

An *in vivo* test was possible in a family which carried both Ho-2 and S hemoglobins Individuals carrying both Ho-2 and S have not only Ho-2 and S hemoglobins, but also a component which moves like A. It was hypothesized that the A component contained hidden within the double hybrid molecule, $(\alpha^{Ho\text{-}2})_2\ \beta^S{}_2$, as well as A. A mixture of $(\alpha^{Ho\text{-}2})_2\ \beta^S{}_2 + \alpha^A{}_2\beta^A{}_2$ should be expected to produce both S and Ho-2. This was confirmed by reassociation experiments with this component.

The attention of the group was next focused on a consideration of "normal" types of hemoglobin other than A Hemoglobin F, fetal hemoglobin, occurs in the human fetus. Its exact time of appearance in the embryo is undecided, but it may be preceded by an earlier type, primitive hemoglobin Hemoglobin F has a molecular structure and configuration basically similar to A in so far as it consists of two α- and two β-chains The α-chains of F and A appear to be similar in amino acid sequence and may in fact be identical. The β-chains, however, are definitely different Since F occurs in A individuals and the structure of the β-chain of F is apparently not affected by β-chain mutations such

as those producing S, it was suggested that the β-chain of F be called the γ-chain and that a γ gene be postulated to control its synthesis This gives three sets of genes, α-, β- and γ-chain genes controlling hemoglobin synthesis in normal individuals

In the normal individual fetal hemoglobin, production ceases soon after birth and is replaced by hemoglobin A The fetal hemoglobin in the newborn seems to occur in mixture with A in some erythrocytes, while some cells may have only A and others only F This would seem to eliminate the possibility that F is produced only in the fetal hematopoietic centers and A in the adult centers

Another normal hemoglobin, A_2, was discussed by Dr Ingram This hemoglobin component can be separated from A by starch block electrophoresis and occurs at levels of about 2 5 per cent of total hemoglobin in normal individuals It is only one of a number of minor components which together probably make up about 10 per cent of the total hemoglobin

A_2 is similar to A except that the part of A_2 which corresponds to the β-chain of A has at least one difference in peptide No 26 Its relationship to A is somewhat like that of fetal hemoglobin to A Both have apparently the same α-chains but the corresponding β-chains are different and furthermore are presumably under the control of different genes Thus, since a γ gene is postulated for the γ-chain of F hemoglobin, it follows that a δ gene should be postulated for the corresponding chain of A_2 which may be labeled the δ-chain

To summarize, there is one α gene postulated for all known types of hemoglobin and a series of genes, β, γ, and δ, for the chains attached to the α-chain in the different "normal" types of hemoglobin

The discussion turned to a consideration of the thalassemias In these inherited diseases, characterized by a microcytic anemia, there are no obviously abnormal hemoglobins present Hemoglobin A and A_2 are present, but there seems to be an increase in A_2 relative to A There are two general types of the disease, thalassemia major and minor The former is thought to be the homozygous and the latter the heterozygous condition In thalassemia major, hemoglobin A is greatly reduced and in a few cases reported has been found completely absent This reduction or complete absence is compensated for, apparently, by the production of fetal hemoglobin

Thalassemia minor can be classified into two categories, those that show an increase in A_2 and those that do not

When thalassemia occurs with hemoglobin S as the double heterozygote, the phenotypic effect can be as severe as in sickle-cell anemia.

Such individuals have hemoglobin S and F and virtually no A A similar result occurs when thalassemia is present as the double heterozygote with C, D, or E The mutant hemoglobin and F are present and A production is suppressed. Dr Ingram proposed therefore that the thalassemia gene specifically suppresses the formation of hemoglobin A.

To provide a mechanism for this hypothesis, he advanced two further hypotheses The first, termed the "substitution hypothesis," proposes that the thalassemia mutations are essentially no different from other mutations such as S, C, D, etc, but that the substitution of a different amino acid in either the α- or β-chain does not lead to an electrophoretic change This would not be expected to interfere with A_2 formation, if the mutation affects the β-chain, but it would be expected to affect A production. A mutation affecting the α-chain would, on the other hand, possibly reduce A_2 formation as well as A, which may explain why some thalassemia minors do not show an increase in the level of A_2. The formation of hemoglobin H, a β_4 hemoglobin, may in fact be the result of a thalassemia gene which so drastically affects the α-chain that insufficient amounts or inadequate chains are formed and the normal β-chains must comprise the tetramers.

The second hypothesis to explain thalassemias given by Dr. Ingram was termed the "tap" hypothesis. This assumes that there are regions of the chromosome that regulate the activity of genes. Thus, the activity of the α-chain gene is controlled by a tap, and in the thalassemic condition this tap permits only a small to zero flow of information from the gene The tap controlling the β gene could also be affected giving another type of thalassemia Dr. Motulsky presented evidence and an hypothesis which suggest that the high S in such cases may be due to a thalassemia in which the β-chain is affected. The *S/thal* heterozygote would therefore be expected to produce an abundance of hemoglobin S if the β-chain produced in the presence of *thal* lost out in the competition for the normal α-chains

Next came a brief discussion of certain aspects of the "dogma." The question asked was, *are there qualitative differences in the primary gene products of a gene depending on the environment?* The general conclusion to this question was no.

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FACTORS MODULATING THE BIOCHEMICAL EXPRESSION OF GENETIC SYSTEMS

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IT IS ALWAYS USEFUL, if you have a belief, to stick as close to it as you can The one useful thing about a dogma is that it can provide the basis for clear unambiguous statements of one's position with respect to relevant issues. Essentially, the dogma may be phrased in the following terms· There is a DNA code which specifies an RNA code which in turn determines the amino acid sequence of a particular protein This concept can be diagrammed as follows.

DNA → RNA → protein (enzyme) → products

In the above, the arrows are presumed to indicate flow of information and the last arrow is meant to signify the products of the enzyme activity The cell must make a very large number and variety of catalysts and it also obviously must regulate the proportionate composition of its enzyme pattern so that normal cell function can occur The question we should like to discuss is what kinds of regulatory mechanisms can and do exist

In terms of the diagrammatic representation of the dogma, one might theoretically expect to be able to insert regulatory devices between each one of the indicated steps Thus, one might conceivably control the rate at which information from DNA is transferred into the RNA code; this would be a device involving the control of gene function Again, one might be able to control the rate at which protein is synthesized by the RNA Finally, one might be able to regulate the activities of existent enzymes These, in essence, are the three possibilities Instances of the last two have been found and studied in some detail Many examples in which controls are exerted at the level of existent enzyme activities have stemmed from Dr Davis's laboratory One example is the pathway leading to the biosynthesis of valine In this, there is a step

involving the condensation of two molecules of pyruvate to acetolactate The activity of the enzyme involved is inhibited by valine. Thus, if the cell is given a pre-formed supply of valine, it will no longer go through the necessary biosynthetic steps by virtue of the inhibition exerted on the condensing enzyme by valine. This poses a problem, because beyond this step, on one branch of the sequence, there is an intermediate which is needed for other purposes. I have asked Dr. Davis to discuss the research which has illuminated this problem

This method of regulation by controlling the activity of an enzyme by the end product of a biosynthetic pathway is generally known as "feedback inhibition."

Davis: As distinguished from feedback repression.

Lederberg: That surely has a much wider connotation than inhibition of enzyme activity.

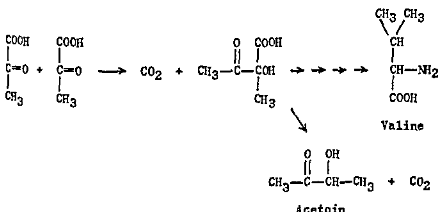
Spiegelman. Feedback inhibition is supposed to denote the ability of one of the products of the sequence to inhibit one of the previous enzymatic steps

The other mechanism of regulation which involves the control of the number of enzyme molecules synthesized is known as "repression" For example, in the case of methionine biosynthesis, the end product will suppress the actual formation of methionine synthetase

Along with repression, there is another phenomenon which historically was discovered and named earlier, "induction" or enzymatic adaptation Operationally, what is meant by induction is the specific stimulation of the synthesis of a given enzyme by the presence of a particular compound which usually is chemically related to its substrate. Compounds having such specific stimulatory capacities are referred to as inducers One of the questions which has been raised by recent experiments, and which I would like to discuss here, is the relation between the phenomena of repression and induction They superficially appear to be distinct but this question must be re-examined in the light of recent investigations The third mechanism, involving control of gene activity and leading to the variation in the number of protein or enzyme-synthesizing machines, remains, at the moment, only a theoretical possibility

Davis: I would like to illustrate some aspects of feedback inhibition and repression with the paths of biosynthesis of isoleucine and valine which Umbarger (10,37) has been pursuing for a number of years At the start, I should like to point out that the experiments I shall discuss, while carried out in my department, were not carried out in my laboratory.

Umbarger has found that the first reaction in valine synthesis is two molecules of pyruvate going to acetolactate; four further reactions yield valine.



Acetolactate was already known as an intermediate in the formation of acetoin, a prominent fermentation product in certain organisms.

In the parallel isoleucine pathway, threonine is converted by threonine deaminase to α-ketobutyrate, which then condenses with pyruvate to yield a compound like acetolactate but with one more carbon This compound is then converted to isoleucine through a series of reactions catalyzed by the same enzymes that act in the valine pathway

In these, as in other sequences, the first step is by definition a branch point. Threonine has other fates, but the conversion to α-ketobutyrate is ordinarily a step only toward isoleucine Similarly, pyruvate has several fates, and of the several metabolic pathways that branch at pyruvate, conversion to acetolactate ordinarily leads only to valine

In these two pathways, as well as in a number of others, it has been shown that for the first (*i e*, the branch-point) reaction the cell makes an enzyme that can be inhibited *in vitro* by addition of the end product of the sequence (*i e.*, isoleucine, valine) This inhibition is highly specific· it cannot be achieved with a variety of metabolically related compounds Similarly, while not all the other enzymes of the sequences have been studied, those that have do not show this susceptibility to inhibition Physiologically, this makes a very sensible picture. If you constructed a cell with substances flowing into a variety of pathways and if you wanted to control these flows, you would put in a tap at each

branch point You would not bother putting in taps every time you coupled a new length of pipe onto an unbranched line.

Such inhibition accounts nicely for the observed feedback control of a biosynthetic process by its own end product. This effect was observed earliest perhaps in a phenomenon long known to people working on biosynthetic pathways with mutants, namely, that mutants blocked in various places could accumulate precursors when grown on a limited amount, but not when grown on an excess, of the required end product It therefore became part of the empirical stock-in-trade of this field, without examination of the underlying mechanism, to grow the organisms under circumstances that provided a limiting supply of the end product in order to get an accumulation Somewhat later, and more systematically, the Carnegie group (27) showed that when a supply of the end product was given that differed isotopically from the endogenous source of the compound, in many cases endogenous synthesis was more or less completely cut off Feedback inhibition provides an explanation for both these findings

The importance of feedback inhibition is underscored by a phenomenon that Umbarger has observed with the first reaction of the isoleucine pathway and also that of the valine pathway. the ability of the cell to produce two different kinds of enzymes to carry out the same reaction I will discuss one of these cases in some detail.

Aerobacter has a major degradative pathway which it uses only when the medium is allowed to become acid Under this circumstance, glucose goes through the usual reactions to pyruvate, which then goes to acetolactate Acetolactate now gets simply decarboxylated, and gives acetoin (see reactions above) This pathway is of obvious value to *Aerobacter*, because acetoin is a nonacidic fermentation product, and the organism produces it under circumstances in which the medium is starting to accumulate enough acid to threaten inhibition of growth

The first reaction in acetoin formation from pyruvate is exactly the same as the first reaction for valine: *i.e.*, the formation of acetolactate It turns out that when the organism is grown under circumstances in which it is making acetoin as a major fermentation product, there is an expansion of the total amount of enzyme that can carry out this conversion of pyruvate But the new enzyme molecules formed differ from those that were formed earlier, when the organism was not making acetoin but was only using this reaction to make valine. The new enzyme differs in a number of respects, such as optimal concentrations of cofactors The two respects that I would like to mention specifically are, first, that the enzyme which functions in the cell when it is making

acetoin, which we will call the degradative enzyme, has a pH optimum of about 2 5 pH units lower than that of the other enzyme Even more *interesting is the fact that the biosynthetic enzyme, as I mentioned* earlier, is inhibited *in vitro* in its activity by valine, and the degradative enzyme is not

Presumably, these two different enzymes correspond to two different genes This means, then, that it is important enough for the cell to keep these feedback messages straight, so that it bothers to use two genes to make batches of enzyme that use the same substrate and make the same product In this way, it avoids the difficulty of having the presence of valine interfere with its making acetoin in an acid medium, or of having an alkaline medium interfere with its necessary manufacture of valine These are, *then, two quite independently functioning systems*

Wagner: Valine does not inhibit the degradative enzyme?

Davis: Valine does not inhibit the degradative enzyme, but it does the biosynthetic Furthermore, valine also represses formation of biosynthetic enzyme, quite a separate process from inhibition of its activity But valine has no effect on the formation of the degradative enzyme Furthermore, as I noted earlier in defining the degradative enzyme, *there are variations in growth conditions that induce or repress* its formation, but these particular conditions do not affect the biosynthetic enzyme, whose formation is controlled by valine

Spiegelman Is there any case where only activity is stopped in this feedback but not synthesis?

Davis: I don't know of any, but I'm not sure this is too fundamental, because there are other cases where mutation has resulted in gain or loss of repressibility by the end product Repressibility is thus something that is subject to mutation With an inhibitable enzyme, I'm quite sure this property should be separable from repressibility

Wagner Are there two batches of this made?

Davis. Yes, two batches are made, depending on how you grow the organism

Wagner As well as a third one for the degradation of acetolactate

Davis Yes This is the next step on the degradative pathway I haven't discussed this, since it is not only a separate enzyme but a separate reaction

Precisely the same thing occurs in the isoleucine pathway as in that of valine The organism can use threonine not only as a precursor of isoleucine but also as an exogenous energy source Under ordinary circumstances, where it is only using the threonine-to-ketobutyrate reaction to make isoleucine, it makes a batch of enzyme that is inhibited

by isoleucine However, when degrading threonine, through the same reaction, it makes a batch of enzyme that is not inhibited by isoleucine.

Furthermore, isoleucine also represses formation of the biosynthetic threonine deaminase but does not repress formation of the degradative threonine deaminase.

I would like to point out an analogy with the hemoglobin problem. Though the genetics of the "alternative" enzymes I've discussed has not been worked out, it seems reasonable to suppose that the two batches of enzyme for a single reaction arise from two different genes; and they certainly arise in the same cell.

I am not sure that the hemoglobin story needs this support, but since there seems to be some skepticism about the possibility that fetal hemoglobin is made in the same cell as adult hemoglobin, I thought I might point out the parallel

In the matter of terminology, you are right, Dr Lederberg, that people working in this field can hardly preempt as broad a term as feedback, which was introduced by electrical engineers But we also find it useful to assign special meanings to other terms which are not the sole property of biologists, such as transduction and recombination.

Lederberg If you added "enzyme activity," it would be all right

Davis: We are trying to be consistent about the use of "repression" to refer to an effect on the manufacture of the enzyme and "inhibition" to refer to an effect on the action of already existing molecules Initially, the words were often used interchangeably and it was quite confusing There is still a source of confusion, because many biochemists like to have specified in any papers concerned with enzymes that an activity has been measured rather than an amount of enzyme, since an amount of a specific protein hasn't been directly measured However, with this convention, when something is spoken of as decreasing the activity of a given enzyme in an extract, it is not clear whether repression or inhibition is meant.

Lederberg You're not entitled to create the distinction between inhibition and repression until you have demonstrated a difference in the amount of protein.

Davis. No, not quite so. You may not know how much of an enzyme protein you have in a test tube; but you can keep it constant and meanwhile change its action by addition of a compound This is clearly inhibition and not repression, as I have defined the terms.

Levinthal. In all cases where there is inhibition by an end product of a sequence, is it always true that the inhibition is on the first enzyme?

Davis: There are several cases where it is the first reaction, branching

off from some common pathway, but not those later reactions in the same sequences that have been studied Repression, in contrast, can affect all the enzymes of a sequence But the phenomenon of repression, which was originally thought to be the probable explanation for the control by an end product of its own synthesis, probably has very little to do with this phenomenon When you put in an end product, you find that it immediately cuts off accumulation of a precursor or cuts off isotopic competition between endogenous and exogenous material Feedback inhibition can account for it, because the effect of the added end product on the action of this enzyme is instantaneous In contrast, any effect on the formation of enzymes in the pathway would not affect biosynthetic activity of that pathway until the pre-formed enzymes became diluted This would be gradual over several generations

It looks as though the phenomenon of repression has as its contribution to the economy of the cell the sparing of the manufacture of unnecessary protein rather than the sparing of the manufacture of unnecessary end products

Wagner: How does Umbarger distinguish experimentally between synthesis and activity?

Davis: The enzyme is measured in extracts *in vitro* To demonstrate inhibition, the activity of a given extract is shown to be less in the presence of the inhibitory end product than in its presence To demonstrate repression, extracts of various batches of cells are tested under identical conditions (*i e.*, in the absence of the end product); cells grown in the presence of the end product (and then washed essentially free of it) are shown to yield extracts with a lower specific activity than cells grown in the absence of the end product You could argue, of course, that the so-called repressed cells contain inhibited enzyme which the test misses However, the results cannot be explained by end-product inhibition, since the amounts of end product that would be required far exceed what could have been carried over from the repressive medium And if you postulate formation of enzyme linked to some tightly bound unknown inhibitor you would no longer be forming "the enzyme", indeed, you would be describing, at a more detailed level than I have attempted, one possible mechanism of repression of enzyme formation

Anfinsen What is the nature of this enzyme interaction on which the inhibition study is done? Is the molecule small, or is it a large, complicated one?

Davis. The phenomenon was first demonstrated with sonicated cells, and how far the purification has proceeded, I don't know.

Anfinsen: Valine is one of the most unlikely inhibitors, structurally and otherwise

Davis: I don't see on what basis there can really be strong preconceptions about it. If it is in the interest of the cell to evolve a mechanism that is going to have two kinds of specificity—specificity with respect to the action that it catalyzes and specificity with respect to permitting its control—how can we set limits on what the controlling compound might be?

Anfinsen: I would like to be convinced, but I need a few more data.

Davis Your feeling is that until it is highly purified, you would wonder? Let's put it this way: Under certain growth conditions, the enzyme formed is not inhibitable by the same product. While an analysis of the mechanism of inhibition at an intimate molecular level is of great interest, would not the phenomenon of specific end-product inhibition be equally valid and significant for the control of metabolism, regardless of its mechanism?

Anfinsen. I was actually thinking of multi-enzyme complexes After all, the ghost of cyclophorase is still with us. Easy observation of effects with complex systems (although more difficult to understand) is more to be anticipated than with more discrete systems.

Davis. Of course, we don't know that the enzymes differ in sequence of amino acids I suppose we are all tempted to make inferences about this You are right that at the present stage one can only conclude that somewhere in the over-all system this inhibition takes place in a way that affects the activity of the enzyme I would have no feeling that it is easier or harder to explain if the difference between inhibitable and noninhibitable enzyme involved the membrane that the enzyme might be attached to or involved the polypeptide chain itself

Spiegelman. Do you recall the levels of valine that were used to get the inhibition?

Davis: The levels are quite low, because Umbarger could actually reverse the inhibition with pyruvate, and the concentrations of the valine he used were considerably lower than the concentrations of pyruvate

Lederberg: How far has the evidence of repression of protein synthesis gone? For example, this problem arises in studying cross-reacting material You use other ways of characterizing the protein besides its activity, because you can always say there is a firmly bound complexant which is inhibiting the activity of a protein that is being made anyway It doesn't have to be the product that you put in Are you

in a position to say for any repressed enzyme synthesis that, in fact, a particular protein is not being made?

Wagner: I can give you a partial answer to that. I have been working with this same system in *Salmonella* If you fractionate young cells that have been grown in the presence of valine and isoleucine, going through the same procedure that you would go through to get out the enzyme, you can't find any sign of the enzyme in the young cells

Spiegelman: A direct answer to your question would be that CRM is repressible, and there you are looking for an immunologically reactive protein

Davis: Doesn't Dr. Levinthal have the best evidence on this with the effect of phosphate on formation of a fraction that can be distinguished by other means besides enzymatic activity?

Levinthal: In the case of phosphatase, exactly the same thing is true. Phosphate inhibits the action of the purified enzyme, and it also represses the synthesis of both active enzyme and the immunological cross-reacting material in those mutants which make CRM but have no enzymatic activity.

Davis So you have zero, in your case

Glass: Do those two forms of the enzyme that you were talking about act on the same step from pyruvate to acetolactate?

Davis: Yes, it involves the same substrates and the same products. The reaction consists in the elimination of CO_2 and formation of a carbon-carbon bond It has not been possible to fractionate the system, but it is conceivable that more than one step is involved in the reaction

Glass· Which destroys another old dogma, doesn't it?

Davis· One enzyme, one gene? I don't know that that ever was an important corollary of the reverse generalization Anyhow, we are talking about *two* enzymes that happen to catalyze the same reaction

Lederberg: If the repression consists of the binding of phosphate tightly to one of two monomeric components of an active polymer, could you exclude that it is necessary to get the polymer before you get antigenic activity? The monomeric units might behave differently chromatographically

Levinthal· Yes, that would be possible

Spiegelman You don't use pre-existing monomers, then?

Lederberg It would be irreversibly discarded

Davis: You are suggesting the picture that most of us probably have, except that the protein doesn't stick to the RNA, something inhibits it and it peels off to become a waste product

Lederberg That's right I think it might be worth while to put in

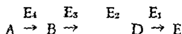
another step here, because we now know that so many proteins are polymers of the monomeric units. We should recognize that anything that gets in the way of polymerization will also get in the way of synthesis of active protein

Davis: Dr Spiegelman has pictured repression of enzyme synthesis as taking place after the coded RNA, which I think it probably does But from the point of view of what we know from the data, it could take place between DNA and RNA. The strongest evidence against it is that you can release it extremely rapidly, almost instantaneously

Spiegelman Those are, in principle, indistinguishable.

Davis You can both start and stop it immediately.

Spiegelman I would like to say a few words about this question which is very puzzling. Let us imagine a sequence of reactions such as follows:



$$A \xrightarrow{E_4} B \xrightarrow{E_3} \quad \xrightarrow{E_2} D \xrightarrow{E_1} E$$

It is not too difficult to imagine how, if one puts in E, one stops not only the formation of E_1 but also the synthesis of all the other enzymes in the sequence One could imagine this as a simple feedback repression proceeding in a chain reaction as follows: putting in E leads to the inhibition of enzyme E_1 and its formation; this, in turn, leads to the pile-up of compound D which then leads to the repression of E_2 and so on down the line However, we now know that isolation of the reaction $B \rightarrow C$ by the insertion of genetic blocks on either side does not lead to a disappearance of its repression by substance E. This makes it impossible to invoke "feedback repression" via a sequence of enzymatic conversions

Anfinsen. Is E_2 and E_3 CRM formed?

Spiegelman. No

Lederberg That is what Dr. Davis was saying; in other words, the repressor represses specifically but does not have a great deal to do with substrate specificity

Spiegelman I don't understand how this mechanism can work

Davis: This means that the end product itself, not through conversion by reversal of the pathway but through its own structure or that of a further derivative of itself, is the direct inhibitor and the direct repressor.

Levinthal. It seems to me worth pointing out explicitly something which has been noted by several investigators, namely, that in all cases so far studied where this linked repression occurs—that is, one small molecule repressing a number of different enzymes—the genes which control the synthesis of these enzymes are genetically linked

Lederberg. This is a consequence of studying these phenomena only in bacteria.

Spiegelman. But neither of these phenomena exists in higher organisms, as far as we know.

Neel: How many systems like this do you know? You say in all cases?

Spiegelman. Tryptophan and cysteine

Levinthal: Histidine

Anfinsen. And maybe tyrosine

Wagner: Multiple repression of enzymes by an end product may occur in many cases However, in *Neurospora* and *Salmonella*, it does not repress the enzymes between acetolactate and end product. This is in spite of the fact that in *Salmonella* the genes controlling these enzymes are closely linked

Spiegelman: And there is no repression?

Wagner. No repression

Davis: Dr Levinthal said that where there is repression of several different enzymes, they are linked Mutants to nonrepressibility can be obtained, and that doesn't change the linkage

Spiegelman: So you are implying that if you have a scatter situation, you wouldn't have this sort of thing You would expect one molecule to do the job of repressing the whole scattered set

Levinthal: The model I am thinking about would suggest this

Smithies Do you think that there is a small section of the polypeptide chain common to all the enzymes, and that the production of this polypeptide is inhibited?

Levinthal. That is one possibility

Lederberg That is not a sufficient requirement for having these genes linked, though

Levinthal. We ought not to press this question of mechanism It is obvious that one can make up a mechanism in which there is a reason for their being linked, if you think of the RNA particle sitting on the DNA

Lederberg But, rather than having some polypeptide chain which is common to all four enzymes, what you just said sounds much more plausible, that there is a continuity of the RNA, the entire segment, not just one functional unit. It is the action of this RNA which is jointly repressed by the soluble repressor

Levinthal: We're really going very far from the evidence The

kind of model which seems to be most consistent with the fact is essentially the one to which Dr. Smithies just referred.

Lederberg. I don't see what that has to do with linkage.

Smithies: There is some evidence in the transferrin system in cattle which may be relevant here Animals heterozygous at the Tf locus have three transferrins; heterozygotes have six.

Spiegelman: Why must there be linkage to have a common sequence?

Levinthal: The reason for thinking of models of this kind was to explain why there is close linkage.

Lederberg: Agreed, and that regulation is the basis of it is almost obvious.

Levinthal: The fact that the addition of repressor or the removal of inducer turns off the synthesis of an enzyme very rapidly would seem to imply that the RNA template is not destroyed and that the inhibition is between the RNA and the protein.

Lederberg. That's all right.

Levinthal· One way of obtaining the control of a number of RNA particles in a cell would be to have them remain attached to the DNA. This could give an automatic control of the maximum number of such RNA particles at say 1 or 2.

Having the RNA templates located physically adjacent to each other might make it easier if several different particles were required to make different peptides in the same protein. In an erythrocyte, this might be unnecessary, because the cell is essentially only making hemoglobin In a bacterium, where probably there are the particles for making a thousand or two thousand different enzymes, the cell is given an advantage if the different peptide producers are physically adjacent.

Lederberg: I do not disagree with anything you have said, but I think you added one bit which is not necessary, that is, that there remains a physical association between the RNA and the DNA. One can make a model in which these closely linked markers are represented in single ribosomes

Levinthal· But many of these sequences are really quite long, and there is no evidence as yet of a ribosome containing that amount of RNA. There are sequences of at least six enzymes in the case of histidine

Smithies: I don't think the number really matters if there is a common element

Levinthal. No, if there is a common element, but if there is one ribosome making six enzymes, it is a question of how much information you can put in one ribosome.

Lederberg: Are these six enzymes all repressible by the same soluble repressor?

Leventhal: Yes

Lederberg. Then how far is it stretching our knowledge of the structure of the ribosome to say that it has the information for all six enzymes? Do you know about these particular ribosomes?

Spiegelman: We know how much RNA they contain.

Hoagland: The molecular weight of the 70 S RNA particles is around 2,000,000 These dissociate into smaller particles, although they probably function as a large particle. We don't know. But that would be enough to make several proteins of average molecular weight. There could be a physical association of synthesizing machinery for several proteins in a single particle

Lederberg: Nothing whatever is known about the distribution of information among particles, is it?

Hoagland: Not a thing

Anfinsen: There is another possibility We might postulate that there is, in each of these enzymes the functions of which depend upon correct secondary and tertiary structure, a constellation of amino acids which has a unique binding affinity for compounds of a certain type The specific compound might induce the formation of the proper three-dimensional configuration by acting, in a sense, as a trapping agent which combines only with protein having a correct spatial configuration. A repressor, then, might be a substance that attaches to this site on the protein during assembly on the template, but which will not cause the protein to fold up, in contrast to the substrate which will actually *induce* the folding All the histidine intermediates just mentioned look somewhat alike and might interact with the same constellation of amino acids

Lederberg How do all the six kinds of ribosomes or ribosome fragments happen to get the same receptor? You would have to put in an extra DNA next to each gene, and then there's no point in having them linked

Anfinsen. It depends on how much you break up the biosynthetic process—how many small intermediates are involved in the assembly of a single polypeptide chain

Leventhal: That's why I want to put in a common peptide.

Lederberg: All right, that's one way The other way is to see whether one has to propose that there is not enough room in the one ribosome for this entire segment of the genetic map

Leventhal. I would like to add one point in connection with Mar-

kert's work (16). When an enzyme from a mammalian system is purified, the Smithies technique of starch gel electrophoresis shows that the enzyme is frequently separable into several active fragments I would like to suggest that these different enzymes or different molecules may have a common sequence for their catalytic activity but a small region of the protein which is different from one to another. This difference could account for where the enzyme likes to sit in the cell. We all know that a large cell is not just a bag of enzymes but that these enzymes are physically located on particular organelles. This being true, a particular enzyme will need two specific sites, one catalytic and one for attachment. It may be that, as with the hemoglobins, one has a common gene for the catalytic site, but several different genes for the different attachment sites.

Spiegelman: One interesting fact in support of this idea is that twenty-four of the proteolytic enzymes do have a common sequence

Lederberg: For the record, will you say which enzymes are linked and have a common repressor?

Levinthal The histidine enzymes in *Salmonella* are the best documented examples I know of. These were recently reported by Ames and Garry (2)

Atwood Are they in different cistrons?

Lederberg They undoubtedly will be.

Atwood. Doesn't this imply that if the ribosomes are detachable, then one cistron, one ribosome? To me, it does.

Lederberg: No.

Atwood Let me explain why I made this comment. Generally, the complementarity of heterokaryons involves different cistrons. If the ribosome is detachable from the DNA and does its protein synthesis elsewhere, there must be two separate species of ribosomes, one from one of the nuclei and one from the other one. Now, you have to repress these independently by the same agent.

Anfinsen. There are seven complementing cistrons in the Ad4 locus.

Smithies: I would like to challenge one thing that has come up; that is the rapidity of repression as evidence for the site of the repression

Spiegelman: Measurements of the order of 4 seconds can easily be made with tracers If the effect is immediate, it is quite clear that it cannot involve only repression of enzyme synthesis.

Davis I think it is necessary to return to these terms *repression* of enzyme formation and *inhibition* of enzyme activity. Inhibition blocks a pathway essentially instantaneously, as is easily demonstrated, whereas the pathway would not stop functioning immediately if further forma-

tion of one or more of its enzymes were simply repressed. But while repression has only a slow effect on a functioning pathway, Dr Levinthal stressed the rapidity of its effect on the synthesis of a protein However, I think it is much easier to demonstrate rapidity of release of repression, because when repression is initiated, there is a background of enzyme already present. Therefore, in the next few minutes, differences of a few per cent are being measured. In contrast, if a cell is already completely repressed and hence has no background content of the enzyme, and that cell is put into a medium which lacks the repressor, rapid synthesis of the enzyme by the "derepressed" cell can be demonstrated within a minute. This is the most striking evidence that the machinery for making protein is already present and becomes active when released from repression It is acting at the normal rate within the shortest time span in which it can be measured

Davis: Yes Technically, things can't be detected quite as fast with repression as with derepression, but the result is more rigorous

Neel Does electron microscopy show enough differences among ribosomes so that there would be some hope of separating them by differential centrifugation or some such technique?

Spiegelman: It has not been done as yet There will be attempts, however, and I believe the techniques are almost with us Density centrifugation, which is one of the most sensitive methods for separating particles, would at present appear to be the preferred procedure for further exploration of such a possibility

Perhaps now I should describe an experiment which is relevant to a comparison of induction and repression We may perhaps start out with a brief background of the nature of the system used, which is the induced synthesis of β-galactosidase in *E coli* The utility of this enzyme system was first pointed out by Dr Lederberg and stems from the fact that it is very easy to assay the enzyme with the aid of a chromogenic substrate such as *o*-nitrophenyl-β-D-galactoside Inducible cells grown in the absence of inducer make very little enzyme, although there is always a low level of detectable activity When the inducer is added, the relative rate of enzyme formation increases sharply and the rate of enzyme synthesis is such as to suggest that a constant fraction of the new protein synthesized goes into the formation of this particular enzyme.

Experiments by Monod and Cohn (23) have demonstrated that non-utilizable analogues of normal substrates of an enzyme can be employed as inducers Such nonutilizable inducers (for example, thiomethyl-β-D-

the z^+ gene enters the z^-i^- cytoplasm it would be coming in contact with an environment under control of the constitutive allele and adequate amounts of the internal inducer should be available. Enzyme synthesis should commence immediately and proceed undisturbed. However, according to the repressor hypothesis, we would expect a somewhat different situation Here, it is assumed that the constitutive cells are constitutive because they lack an internal repressor and that the inducible cells are the ones which possess it When the z^+ gene enters the constitutive cytoplasm, it will, therefore, start to make enzyme because of the absence of the repressor However, because of the fact that the i^+ gene is linked closely with it, the i^+ should begin to function and gradually build up concentrations of repressor which will eventually suppress the activity of the z^+ locus This is what is observed As a further check on the adequacy of this explanation, one may consider the mating of the following type:

$$♂(z^-i^-) \times ♀(z^+i^+) \qquad \text{(C)}$$

but again in the absence of inducer. Such matings reveal no trace of enzyme at any time after mixing It will be noted that they are of the same constitution as the mating (B) described above. However, in mating (C), no enzyme synthesis would have been expected according to the repressor hypothesis since the z^- gene is entering the cytoplasm under the control of the i^+ locus. It may be further noted that after enzyme synthesis has ceased in the (B) mating, introduction of inducer immediately leads to the onset of further enzyme formation

Another very interesting question was asked with the aid of inhibitors of protein synthesis. A mating of type (B) was carried out, but an agent such as 5-methyltryptophan was included for various periods of time and then the inhibition released by adding excess tryptophan Of course, in the presence of this agent, the formation of β-galactosidase would not occur, but the interesting question is whether the i^+ gene can function to make its product. This would serve to answer the question whether the repressor was either protein or a product of a newly formed protein If it were either one of these two, its appearance should be prevented by the 5-methyltryptophan On reversal of this agent, one would expect simply to reproduce the entire phenomenon displaced some minutes along the time scale. However, if the repressor is not a protein and does not require the synthesis of a protein for its formation, the period of preincubation in the presence of the 5-methyltryptophan should lead to the accumulation of the repressor. This would be reflected in a lower capacity to synthesize β-galactosidase on

reversing the protein-inhibiting effect of the 5-methyltryptophan When such experiments were performed, it was found that the inhibition of protein synthesis for 60 minutes in the presence of 5-methyltryptophan did not prevent the accumulation of the i^+ gene product. These experiments are interpreted at the present time to indicate that the repressor is neither a protein nor does it require the synthesis of a new protein for its own formation The most likely candidate envisioned now is one which is most exciting, and that is a direct gene product, a nucleic acid

Lederberg But why can't it be a polysaccharide or a sugar or something of that sort?

Spiegelman. To make a polysaccharide, a pre-existent protein is required

Atwood. Did he get the same result in the reciprocal cross?

Spiegelman: Yes The possibility that the repressor is RNA can be tested with 5-hydroxyuridine, and the answer should be obtained very quickly

Lederberg What is the possibility that 5-methyltryptophan is not inhibiting the synthesis of the requisite protein in this case?

Spiegelman. It is still possible that it is making, say, one molecule per cell, which if enough to do the job, could not be readily eliminated

Smithies Does it depend upon the tryptophan content of the final protein as to whether 5-methyltryptophan is an inhibitor?

Spiegelman: If you had a protein which didn't need tryptophan, you might not be able to inhibit its synthesis with this agent There are proteins which do not contain tryptophan

Smithies So the substance concerned could perhaps be a protein lacking tryptophan?

Spiegelman The experiment was first done with chloramphenicol The same answer is obtained, but it is less certain because you can't readily reverse chloramphenicol inhibition of protein synthesis

Smithies I would think it very important to eliminate the possibility of a protein's being involved.

Spiegelman There is one other feature about these experiments which I think one must be aware of, namely, the speed with which the z^+ gene expresses itself It is as fast as you can measure it The rapidity with which synthesis begins is rather disturbing if ribosomes have to be made as a prerequisite

Lederberg It has got to replace the existent ribosome population, though, to get this rate of formation The same initial rate of formation results as with a fully induced cell

Spiegelman All that means is that you have to make one ribosome

How do we know a ribosome is not going in attached to the RNA? M Riley, A B Pardee, F. Jacob, and J. Monod (26) performed an experiment the nature of which is as follows An Hfr (z^+) is labeled with P^{32} and then mated with an unlabeled $F^-(z^-)$ The z^+ is allowed to make some enzyme molecules Then the zygote is frozen and the P^{32} allowed to decay We ask, does the cell still need the original gene that was put in to make more enzyme molecules, even after it has learned how to make an enzyme molecule? It turns out that the enzyme-forming ability decays precisely along with the P^{32} So the information which is injected has not been transferred to anything which is not labeled with P^{32}

Smithies You may have transferred the information, but it isn't stable in the absence of the gene

Spiegelman: That's right It is not something you can use over and over again

Lederberg: Hasn't it been found that a break anywhere in the DNA would prevent new enzyme synthesis? The kinetics were such that the break in the DNA chain did not have to be in the *lac* region to inhibit synthesis, although during a mating this doesn't stop it immediately

Spiegelman· That experiment remains as a wonderfully isolated thing

Lederberg· Suppose you break the DNA in the Hfr cell and then you do know that you can transfer the proximal segments before the break to the F^- Does it then become active again?

Spiegelman You mean, combine the Jacob-Wollman experiment with this? I don't know whether anyone has done it or not. It's worth doing

Lederberg Because breaking the DNA after it is transferred does inhibit it, apparently It doesn't matter where it is broken

Spiegelman· If we could be sure that no ribosomes are transferred during the mating, we would have to say that there is no stable intermediate between DNA and whatever material it uses to make the copy. Any ribosomes that would be transferred would also be hot and would also decay

Lederberg What is the maximum amount of RNA that could be transferred during mating in this experiment?

Levinthal. Their experiments were not set up to detect RNA.

Spiegelman There are all kinds of factors to be considered, such as enzyme getting over during the mating, and so on Jacob and his group did some good control experiments. For example, they asked the question, supposing one mates an Hfr which is arginine-sufficient with an

F^- which is arginine-deficient. The arginine locus is located so far away that it doesn't enter the F^- Can the F^- make β-galactosidase in the absence of added arginine? Had arginine or the requisite synthetic enzymes got into the F^- during the mating, the answer, of course, would be "yes" But the answer very definitely is "no." No detectable amount of cross-feeding occurs in such experiments, although several different arrangements were tried At present, it doesn't seem likely that appreciable numbers of ribosomes are transferred passively with cytoplasmic components during mating

Levinthal: Let's leave them attached to the DNA

Davis: I don't see that the transfer of ribosomes is so critical What you are concluding, I assume, is that after this gene has been transferred into the F^-, it does not make a stable ribosome which then resists P^{32} decay

Lederberg: In these experiments can you exclude P^{32} decay in the RNA as well?

Davis But the P^{32} incorporation took place before the mating.

Levinthal But if you transferred to ribosome, that ribosome would be P^{32}

Davis: If you're making new ribosome?

Levinthal. No Suppose, for example, that a gene could only make one or two ribosomes, and this number arose from the fact that the ribosome was sitting on the DNA Then the DNA transferred with the ribosomes attached can't make any new ones, because the sites for making them are already occupied This ribosome can be hot and decay and that would be the end of it

Lederberg: Is it known whether any RNA synthesis is required in the F^- cell for zygotic enzyme synthesis to take place?

Spiegelman No, that was not tried It could be done with 5-hydroxyuridine.

Lederberg That could almost be the critical experiment for this purpose.

Spiegelman I would be very surprised if it didn't, because every time we have succeeded in stopping RNA synthesis with an agent which really cuts it out, we have always stopped induction

Lederberg Because you are not merely stopping ribosome initiation from the DNA but the rest of the assembly.

Spiegelman I would like the ribosomes to be attached to the DNA, and then I wouldn't worry about the problem of the speed with which things get started That would be good

Hoagland: Isn't there information available on whether RNA does go across?

Spiegelman: No I haven't heard of a good experiment designed for it

Lederberg: Undoubtedly, it will be done, just like the separation of microsomes

Atwood. Would there be a way of making a z^+ from two z^- units that were mutants in the same cistron? After the cross, the one that comes in can't bring a ribosome, because it can't make one that's any good.

Spiegelman: How about ribosomal complementation?

Lederberg. It shouldn't happen It would be cistronic, which means it is stable in the ribosome

Atwood: The only trouble is, there wouldn't be very many recombinant z^+ cells, and you would have to look at the enzyme coming out of just a few of all the cells.

Lederberg: What would you find out, then?

Atwood: Whether it suddenly starts to make enzyme when the recombinant appears

Lederberg: It will, undoubtedly, but then what?

Atwood The experiment is comparable to those we have discussed, except that it avoids the possible introduction of a functional ribosome The i^+ would be carried in

Lederberg: Then, it will not start making enzyme because the i^+ has had 2 hours

Atwood: Yes, so it's a question of the race between the i^+ and the recombination The experiment is probably worthless in any case, a short delay in enzyme synthesis, representing the time required to form a ribosome, would be undetectable.

Lederberg: One reasonably clean case of a No. 3 regulating mechanism (variation in number of protein-synthesizing machines) is antibody formation. In the secondary response, the lymph node is composed of many cells which are not basophilic and which have a small number of the particles we now associate with protein formation. Over a period of 3 or 4 days, these cells are transformed into plasma cells, highly basophilic, full of ergastoplasm. I don't think there is any other possible interpretation

Neel: It would be interesting for those who work with bacteria to consider some of the results Dr. Marks has been getting with his clinical material.

Marks: Glucose-6-phosphate dehydrogenase (G-6-P. D.) deficiency

provides a model for studying factors which may modulate the biochemical expression of a genetic system in man A deficiency of this enzyme in erythrocytes was recognized by Carson *et al.* (6) to be a predisposing factor to the hemolytic anemia that may follow primaquine administration Subsequently, G-6-P D deficiency was recognized to be widespread in its incidence, and it was also recognized that a large variety of agents could induce hemolysis in subjects with this trait (4,19) Table II indicates some of these agents.

Motulsky: What is the evidence on the viral infections?

Marks. That viral infections may induce acute hemolytic anemia is suggested by the occurrence of acute hemolysis in association with viral infections in persons with the enzyme deficiency

Motulsky: Have you seen such cases?

TABLE II

Agents Reported to Induce Hemolytic Anemia in Subjects with Glucose-6-Phosphate Dehydrogenase Deficiency (3,4)

Primaquine	Fava Bean
Pamaquine	Sulfoxone
Sulfanilamide	*p*-Aminosalicylic acid
Sulfapyridine	Infectious mononucleosis
Sulfisoxazole	Viral respiratory infections
Nitrofurantoin	Viral hepatitis
Acetanilide	Bacterial pneumonias and septicemias
Methylene blue	? Typhoid
Phenylhydrazine	? Vitamin K derivatives
Acetophenetidine	? Aspirin
Naphthalene	? Isoniazid

Marks: What appear to be hemolytic anemias induced by a viral infection in subjects with G-6-P. D. deficiency have been seen in our clinic* and by the group in Israel (35). We have observed four such cases in association with viral hepatitis and three with infectious mononucleosis.

I have placed question marks before certain of the agents listed in Table II. This is to indicate that, though these drugs are in common use, reports of hemolytic anemias following their ingestion are rare.

Many investigators have reported that subjects with erythrocyte G-6-P. D. deficiency may be exposed to potentially offending agents without necessarily developing acute hemolytic anemias (4,19). We do not know the mechanism or mechanisms by which a deficiency of G-6-P. D. in the erythrocyte predisposes to acute hemolytic anemia.

The incidence of G-6-P. D. deficiency varies markedly among different population groups. The highest incidence of this trait has been reported among the Jews from Kurdistan (32), where the observed frequency is 50 to 60 per cent. In this regard, the studies of the Israeli group (33) are particularly interesting for they have had an opportunity to examine several Jewish populations of different ethnic origins. In addition to the high incidence among Kurdistani Jews, the frequency of the trait exceeds 20 per cent among Iraqi Jews, but is as low as 2 per cent among North African Jews, and even rarer among Ashkenazic Jews. Studies in this country (1,8,9) have shown an incidence of this enzyme deficiency of approximately 15 per cent among Negroes. The trait is relatively frequent among Caucasians of Greek and Sardinian extraction (28). In addition, the defect has been found among Javanese and certain South American Indian tribes, the Carib and Oyana groups.*

In both Caucasian and Negro populations, a considerable amount of data have been accumulated to indicate that this enzyme deficiency is probably the result of a sex-linked gene of intermediate dominance (8,9,32). The trait is expressed as a very marked deficiency in G-6-P. D. among affected males, while affected females generally have intermediate levels of the enzyme. These observations are consistent with the hypothesis that affected males are hemizygotes and affected females are predominantly heterozygotes.

It has been assumed that the deficiency in G-6-P. D. in various population groups is a result of the same gene mechanism. The explanation of the varied incidence of this enzyme defect in different *racial groups*

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*Marks, P. A.: Unpublished observations.

has remained an intriguing problem Recently, evidence has been obtained to indicate that this trait may not be genetically homogeneous (20). Erythrocyte G-6-P. D activity of affected Caucasian males is significantly lower than that of affected Negro males (Table III) In a number of Caucasian males, we could detect no erythrocyte G-6-P D. activity. Affected Negro and Caucasian females do not differ significantly with respect to the degree of erythrocyte enzyme deficiency Presumably, this reflects the fact that affected females are predominantly heterozygotes. The presence of a single normal gene could be sufficient to mask differences in enzyme activity attributable to the mutant genes

In addition to a difference in the severity of G-6-P D deficiency in erythrocytes, affected Caucasian males have markedly lower levels of this enzyme in their leukocytes than do affected Negro males (Table IV).

Thus, there is a variation in expression of this trait among Negroes compared with Caucasians Further, the expression of this enzyme deficiency is modified by cellular environment This is indicated by

TABLE III

Erythrocyte Glucose-6-Phosphate Dehydrogenase Activity Among Subjects with a Deficiency of this Enzyme

Subject	No	Erythrocyte G-6-P D *	
		Mean	S D
Males			
Negro	59	2 7	±1 5
Caucasian	22	0 4	±0.4
Females			
Negro	52	6 9	±2 3
Caucasian	22	6.8	±3 2

*Enzyme activity is expressed as change in optical density units per minute per gram hemoglobin The mean normal value is 16 1 ± 2 2 OD/min/gm Hb

TABLE IV

Glucose-6-Phosphate Dehydrogenase Activity in Leukocytes of Subjects with Normal and Low Levels of this Enzyme in Their Erythrocytes

Subjects	No.	Leukocytes*	Erythrocytes†
Control	90	46 7	15 1
Affected			
Negro	43	42 1	2.0
Caucasian	20	15.5	0 3

*G-6-P D activity is expressed as change in optical density units per minute per 10^9 leukocytes
†G-6-P D activity is expressed as change in optical density units per min per gm hemoglobin

finding that, in affected Negroes and Caucasians, the decrease in G-6-P D activity is more marked in erythrocytes than in leukocytes.

In considering the possible explanations for the variation in expression of this trait, an attempt has been made to gain insight into the mechanism by which the mutation leads to the enzyme deficiency In subjects with normal G-6-P D. levels, the activity of this enzyme decreases with *in vivo* aging of erythrocytes (22). In mutant subjects, it is possible that an enzyme is formed which is altered in such a manner that its activity decreases more rapidly than normal as erythrocytes age *in vivo*. In erythrocytes, which may synthesize no protein, this type of alteration could lead to a more marked deficiency of the enzyme than in tissues which do synthesize proteins. If this hypothesis is correct, a comparison of G-6-P D activities in young and in old erythrocytes might be expected to reveal greater differences in activity among affected subjects than in normal ones. Such a study was attempted by assaying the enzyme activity in young and old erythrocyte fractions separated by a method of serial osmotic hemolysis (20). By employing Fe^{59} labeling *in vivo* to date the age of erythrocytes, it has been found that this method achieves a partial separation of young and old cells such that

the osmotically resistant fraction is enriched with young cells and the osmotically fragile fraction is enriched with old cells

The mean value for enzyme activity in the younger erythrocyte fractions exceeded that in the older cells by a factor of 7 7 for the control group (Table V). In Negroes with a marked erythrocyte G-6-P. D. deficiency, enzyme activity in young cell fractions, while not approaching that of the controls, was 6 9 times higher than in the old erythrocyte fraction Caucasians with very low erythrocyte G-6-P. D had little detectable erythrocyte activity in the younger cell fractions and none was detectable in the older cells

Lederberg Were the Fe^{59} determinations done on your subjects, or are those data based on the general experience with this method? There is differential hemolysis, isn't there?

Marks. Among the subjects included in the data summarized in Table V, Fe^{59} studies were performed on eight of the controls and four of the affected Negroes. The values indicated in Table V represent mean values obtained in studies on a total of nineteen control subjects, eleven affected Negroes, and ten affected Caucasians (20) However,

TABLE V

Glucose-6-Phosphate Dehydrogenase Activity in Young and Old Erythrocytes Separated by Differential Osmotic Hemolysis

Subjects	WEP*	Erythrocyte Fraction	
		Young	Old
Control	15 6	34 7	4 5
Affected			
Negro Males	2 4	6 2	0 9
Caucasian Males	0 5	1 0	0 0

*WEP is whole erythrocyte population
Enzyme activity is expressed as OD/min/gm Hb

enzyme data obtained in subjects in whom the erythrocyte fractionations were validated by Fe^{59} studies are comparable to the group as a whole

Lederberg: But is there some variability in fragility, independent of age, which is correlated with the enzyme content in the cells?

Marks There is none that we have detected

Lederberg. The iron covers this?

Marks. The studies on the effect of age on erythrocyte enzyme activity suggest that G-6-P D may be a critical factor in determining the life span of the erythrocyte (15,22). The possibility exists that the activity of G-6-P D., perhaps through its function in generating reduced TPN, may be critical in determining the osmotic fragility of the erythrocytes

The data obtained with regard to the effect of age on erythrocyte enzyme activity are inconclusive as to whether or not, in affected subjects, G-6-P. D activity decreases more rapidly than normal as the cells age In many of the affected Negroes and all of the affected Caucasians, the enzyme activity in the young cell fractions was very low Thus, these data do not exclude the possibility that, in affected subjects, a more rapid than normal decrease in G-6-P. D. activity occurs during pre-circulatory erythrocyte maturation, or the very earliest days of the life span of the erythrocyte in the circulation. In this case, the enzyme activity in newly formed nucleated erythrocytes might be normal, but the level in mature circulating erythrocytes, even in young ones, would be low In normal subjects, the level of G-6-P. D in nucleated erythrocytes may be about 100-fold (14) and in reticulocytes about 5-fold (20) that in the nonnucleated erythrocyte cell of average age. Thus, in mutant subjects, the decline in enzyme activity may still be linear, but G-6-P D activity would reach a low level in relatively young erythrocytes, and the differences between young and old cells in mutant subjects might even be less than that in normal

Sutton. Is that really linear rather than monomolecular?

Marks It is monomolecular.

Lederberg: On what evidence?

Marks: Löhr *et al* (15) have provided evidence that the decrease in G-6-P. D activity with erythrocyte aging *in vivo* is monomolecular. These workers determined G-6-P. D. activity in transfused donor erythrocytes at varying intervals up to about 90 days following their administration. It was found that the activity of this enzyme decreased exponentially.

Lederberg: That is, a per cell fashion?

Marks: Yes, they found that the enzyme activity per cell decreased On the basis of our studies employing techniques of graded osmotic hemolysis and differential centrifugation to separate reticulocytes, young mature erythrocytes, and old cells, it is possible to estimate the G-6-P. D activity at three different points in the life span of the erythrocytes in normal and mutant subjects. Such data permit evaluation of whether or not the decrease in enzyme activity with cell aging is monomolecular (20,22) On the basis of such an analysis, it would appear that the decrease in G-6-P D activity decreases in an exponential fashion in both normal and mutant subjects However, in view of the great variability among different subjects, these data cannot permit accurate quantitative evaluation of the relative rates of the exponential decline in G-6-P D activity in normal and mutant subjects Such an analysis may be possible employing techniques like those used by the German workers (15)

I will now briefly review the studies to determine whether an altered enzyme is formed in mutant subjects Several laboratories have reported that G-6-P D of affected subjects, compared with that of normal subjects, has a greater heat lability upon incubation of crude hemolysates at 37°C (7,21,24) (Figure 35) On the basis of these observations, it has been suggested that the mutant enzyme may be less thermostable than the normal enzyme

Evidence has been obtained which indicates that, at least in affected Negro subjects, the enzyme is not different in its thermostability from that of normal individuals (17,18) Thus, a comparison of G-6-P D activity in lysates of leukocytes of normal and affected Negroes incubated at 37°C revealed no differences in the rates of decline in enzyme activity (Figure 35) On the other hand, in the lysates of leukocytes of affected Caucasians, G-6-P D activity decreased to undetectable levels after incubation for 2 hours The apparent decreased stability of G-6-P D in erythrocytes and leukocytes of affected Caucasians and in erythrocytes of affected Negroes correlates with initial low concentrations of the enzyme in the crude cell lysates

In addition, studies with purified normal and mutant G-6-P D have revealed no difference in thermostability G-6-P D was purified from erythrocytes of normal and affected Negro males, employing ammonium sulfate fractionation, followed by adsorption on calcium phosphate gel, elution in a phosphate buffer, and then a second series of ammonium sulfate precipitations* The 40 to 50 per cent ammonium sulfate fraction has G-6-P D with a specific activity 500-fold that of the crude

*Marks P A Szeinberg, A, and Banks J Unpublished data

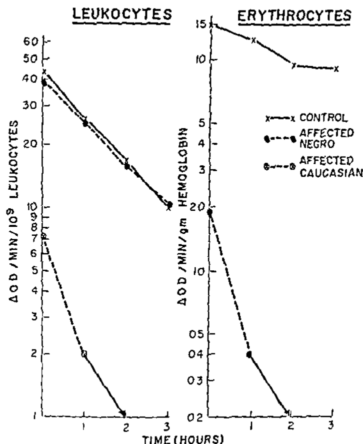


FIGURE 35 The stability of G-6-P. D. (glucose-6-phosphate dehydrogenase) in leukocyte lysates and crude hemolysates during incubation at 37°C No. G-6-P D activity was detectable in erythrocytes of the affected Caucasian The arrow indicates that the enzyme activity fell to an undetectable level (21)

hemolysate in a yield of about 40 per cent This preparation is essentially free of hemoglobin, 6-phosphogluconic dehydrogenase, or hexokinase

Normal and mutant purified G-6-P. D., when incubated at 37°C in concentration about 10-fold that of erythrocytes, showed an increase in activity (Figure 36) Dilution of the enzymes was associated with a loss of this activatability. Indeed, the stability of the G-6-P. D. decreased with dilution These findings suggested that a dissociable factor was necessary to the stability of the enzymes Upon serial dilution, the decay in enzyme activity reached a maximal rate, which was first-order

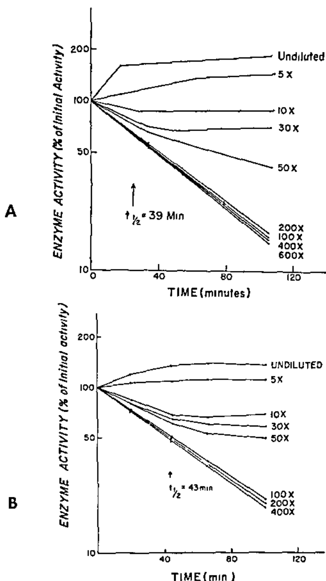


FIGURE 36 The effect of dilution on the stability of G-6-P D (glucose-6-phosphate dehydrogenase) purified from erythrocytes of normal (*A*) and mutant (*B*) subjects The enzyme was diluted in water and incubated at 37°C The numbers at the right indicate the extent of dilution Enzyme activity was determined at intervals as indicated by the points The $t_{1/2}$ is the half time for decrease in activity when the enzyme is diluted to the point where a maximal rate of decay was observed

in its kinetics The half-times of decay were similar for normal and mutant enzymes (Figure 36).

The possibility that triphosphopyridine nucleotide was the dissociable factor necessary to stability of G-6-P D was suggested by the fact that G-6-P. D activity in crude hemolysates was stabilized by addition of TPN in concentrations as low as 5 x 10^{-7} M (18).

Lederberg: Is this the coenzyme?

Marks. Yes *This enzyme has two substrates, glucose-6-phosphate* and TPN We had other observations which suggested that TPN might be necessary to the stability of G-6-P. D Upon incubation at 37°C, G-6-P D is more stable in stroma-free hemolysate than in full hemolysate, or in stroma-free hemolysate to which stroma is added (7) Further, in full hemolysates or stroma-free hemolysates plus stroma, G-6-P. D of normal enzymes is more stable than that of mutants This is the case even when the normal stroma-free hemolysate is diluted so that the G-6-P D activity is at the mutant level and then stroma added.* The stroma inactivation of normal or mutant G-6-P D. is inhibited by addition of TPN, TPNH, or nicotinamide (18).

In view of the TPN protection of G-6-P D. in crude hemolysates, a study was made of the effect of varying concentrations of TPN on the stability of purified normal and mutant G-6-P D. incubated at 37°C. (Figure 37) It was found that with increasing concentrations of TPN (or TPNH), 1 x 10^{-8} M to 4 x 10^{-6} M, there was a progressively increasing stabilization of the normal and mutant enzymes DPN did not stabilize the purified preparations of G-6-P. D. From these data, a concentration of TPN can be calculated at which half maximal stabilization is achieved. This value was similar for the normal and mutant enzymes, *i e.*, 4 x 10^{-7} M This is a concentration of TPN about one order of magniture less than the Michaelis constant for the affinity of the enzyme for TPN at its catalytic site

Lederberg: Do you incubate these preparations with excess TPN for some interval before you assay them?

Marks No The enzyme is assayed in the presence of a large excess of TPN. The assays are done immediately upon removal of the aliquot from the enzyme solution being incubated at 37°C. (Figure 37).

Lederberg: I suppose it is just a slow dissociation of TPN from the enzyme How do you measure the Michaelis constant for TPN for the enzyme activity?

Marks: That is done by determining the effect of varying the concen-

*Marks, P A, Szeinberg, A, and Banks, J Unpublished data.

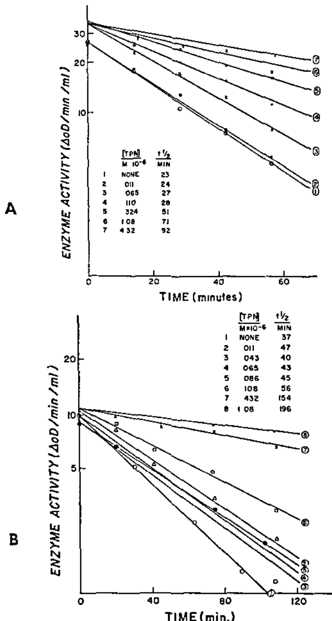


FIGURE 37 The effect of varying concentrations of TPN on the stability of normal (*A*) and mutant (*B*) G-6-P D (glucose-6-phosphate dehydrogenase). The enzyme was diluted in water so that the rate of decay in activity was maximal (curve 1) The enzyme, so diluted, was incubated at 37°C. with varying concentrations of TPN as indicated for curves 2 through 7

tration of TPN on the activity of G-6-P. D. in an otherwise constant reaction mixture.

Lederberg: How long do you incubate them before you add the substrate?

Marks: In determining the Michaelis constant for the affinity of G-6-P D. for TPN, we do not preincubate the enzyme mixture To evaluate the effect of TPN on the stability of G-6-P. D., the enzyme is incubated with the TPN for variable periods of times prior to assay of the enzyme as indicated in Figure 37.

Figure 38 indicates the affect on G-6-P D stability of addition of TPN at varying intervals during incubation of the purified enzyme at 37°C G-6-P D. activity was essentially completely stabilized by addition of 5×10^{-8} *M* TPN at zero time. Addition of this amount of TPN

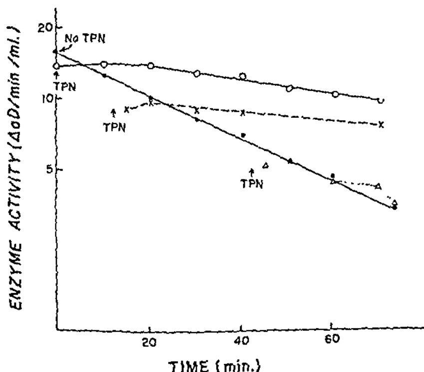


FIGURE 38 The ability of TPN to stabilize and reactivate inactivated G-6-P D (glucose-6-phosphate dehydrogenase) The enzyme was diluted so that its rate of decay was maximal during incubation at 37°C. At zero time, 20 min and 50 min, TPN, in final concentration of 5×10^{-8} M, was added to different samples of the incubating enzyme The control represents the enzyme incubated without added TPN

at a subsequent time, within limits, stabilized the remaining enzyme activity, but caused little or no reactivation of inactivated enzyme. Apparently, the loss in enzyme activity during incubation at 37°C involves a denaturation of the enzyme.

These data support the concept that there is TPN bound to normal and mutant G-6-P D. Upon dissociation of the cofactor, the enzyme becomes unstable and denatures. Further evidence that the purified normal and mutant enzyme preparations contain bound pyridine nucleotide is the finding that they have fluorescence absorption and emission maxima which correspond to that of free TPNH *

The normal and mutant purified G-6-P D do not differ with regard to affinity constants for TPN, G-6-P, acetylpyridine TPN, thionicotinamide TPN or nicotinamide, pH optima or Versene inhibition (Table VI) (17) * Kirkman (12) has also found that the normal and

*Marks, P A, Szeinberg A, and Banks, J Unpublished data

TABLE VI

Properties of Glucose-6-Phosphate Purified from Erythrocytes of Normal and Mutant Subjects

Property	Source of G-6-P D	
	Normal	Mutant
K_m — TPN	4 2 x 10^{-6}M	3 5 x 10^{-6}M
G-6-P	3 5 x 10^{-5}M	3 0 x 10^{-5}M
K_i — APTPN	3 6 x 10^{-5}M	7 0 x 10^{-5}M
ThioTPN	2.5 x 10^{-5}M	1 6 x 10^{-5}M
Nicotinamide	9 1 x 10^{-2}M	6.5 x 10^{-2}M
pH optimum	8 to 9	8 to 9

TPN = triphosphopyridine nucleotide
G-6-P = glucose-6-phosphate
APTPN = *acetylpyridine analogue of TPN*
ThioTPN = thionicotinamide analogue of TPN

mutant enzymes are similar with regard to their catalytic properties. In addition, we have studied the electrophoretic mobility of the enzyme *in starch gel employing a borate buffer, pH 8.5. A* technique similar to that described by Markert and Møller (16) was used to identify G-6-P D specifically. Purified normal and mutant enzymes and the enzyme in crude hemolysates of normal and mutant subjects had similar mobilities

In other studies, the possibility has been examined that G-6-P. D. deficiency reflects the presence of an inhibitor or the absence of an activator in mutants Experiments with mixtures of crude hemolysates from normal and mutant subjects have revealed no evidence of either stabilization of the mutant enzyme or increased inactivation of the normal enzyme Further, normal or mutant purified enzyme added to erythrocyte stroma of normal or mutant subjects is inactivated at similar rates*

In summary, the mechanism by which the mutation in man causes a deficiency in G-6-P D has not been defined The enzyme in crude hemolysates of mutant subjects is less stable than normal This difference in stability of G-6-P D is not detectable upon purification of the *enzyme from erythrocytes of normal and mutant subjects* Bound TPN is necessary to the stability of normal and mutant G-6-P. D. There is a factor in normal and mutant stroma, probably a TPNase, which can specifically inactivate G-6-P D Mutant and normal subjects have not been found to differ qualitatively or quantitatively with regard to this stroma factor The decreased G-6-P. D. activity in mutants could reflect impaired synthesis of the enzyme. However, our thinking at the moment is that the mutation involves an alteration in a factor involved in the stability of G-6-P D, perhaps by decreasing the availability of TPN. Such a defect would be compatible with the observations that the difference in stability between normal and mutant enzymes in crude hemolysates is not detected after purification of the enzymes

Neel. Would you say something about the other enzymatic differences encountered in association with this defect?

Marks G-6-P. D deficient erythrocytes appear to differ from normal ones with respect to several biochemical parameters (4,19). The concentration of reduced glutathione in erythrocytes of drug-sensitive subjects is generally lower than that of normal persons Beutler (3) has found that, following *in vitro* incubation with acetylphenylhydrazine, mutant erythrocytes but not normal erythrocytes show a marked decrease in the concentration of reduced glutathione. The rate of

*Marks, P A, Szeinberg, A, and Banks, J · Unpublished data

glycine incorporation into glutathione of mutant erythrocytes was found to be lower than in normal cells (31) It has been found that mutant erythrocytes have increased concentrations of the enzymes glutathione reductase and aldolase (30), decreased concentrations of catalase (36), and increased concentrations of the cofacter TPN (29). In conflict with these findings, are reports of normal levels of catalase (5,34) and aldolase (11) and evidence of decreased levels of total TPN plus TPNH (13) We have not compared TPN concentration in normal and mutant erythrocytes G-6-P D deficiency might reflect an alteration in the TPN-dependent stabilization of G-6-P D. In normal and mutant erythrocytes, there appears to be a competition between the enzyme and some other factor for TPN

Lederberg Which you already named?

Marks Yes, a factor in stroma does inactivate G-6 P D probably by action on the bound TPN However, we have not elucidated the mechanism of the difference between normal and mutant cells with respect to this system

Smithies. Do they have any variation in stability to oxidation?

Marks: The stability of the enzyme has not been systematically studied in this regard

Smithies. There could conceivably be a difference in the sulfhydryl groups in the two proteins which would affect their relationships with glutathione and catalase but have no effect on their electrophoretic mobilities

Marks This is a possibility

SUMMARY

Wagner Dr Spiegelman opened the discussion of the main topic—the general problem of regulation of the activity of those proteins produced by the operation of the series of events postulated by the dogma Four mechanisms are suggested which may be involved the inhibition of activity of an enzyme by one of the products of that enzyme's activity —the feedback mechanism by inhibition; the actual repression of synthesis of an enzyme by what is normally its primary product or a derivative of its product—feedback repression as distinct from feedback inhibition; the stimulation of appearance of an enzyme by a specific compound—induction, and the mechanism controlling the number of protein-synthesizing machines The last is a hypothetical possibility, whereas the first three mechanisms have an experimental basis in fact.

Dr Davis illustrated the first mechanism—feedback inhibition—with

a description of the work of Umbarger on the biosynthetic pathway leading to the formation of valine and isoleucine in *E. coli.* In this organism, it can be shown that the presence of free valine inhibits *in vitro* the activity of an enzyme which catalyzes the production of its precursor, acetolactate, from pyruvate The bacterium *Aerobacter aerogenes* has a similar pathway leading to the formation of valine from pyruvate However, when it is grown under acidic conditions, it forms a second enzyme which not only produces acetolactate from pyruvate, but also decarboxylates the acetolactate with the formation of CO_2 and acetylmethylcarbinol This enzyme is not inhibited by valine Thus, valine is quite specific in effect; it inhibits only the enzyme which produces acetolactate for valine formation, not the one which presumably uses up excess pyruvate to produce neutral acetylmethylcarbinol. Not only does valine inhibit the activity of the pyruvate-condensing enzyme, but also it inhibits its synthesis Therefore, it is both a feedback inhibitor and repressor.

In certain organisms, it would appear that the end product may repress a number of different enzymes involved in its synthesis Ordinarily the genes which control the synthesis of these enzymes are closely linked

The discussion turned then to the pajama experiment of Pardee, Jacob, and Monod (25). This experiment was an attempt to clarify the phenomenon of enzyme induction, and was discussed by Dr. Spiegelman as one aspect of the analysis of regulatory mechanisms

This experiment involved the use of *E. coli* Hfr and F^- strains which carried various combinations of two genes found to be important in the synthesis of the enzyme β-galactosidase. The gene z^+ apparently directly controls the synthesis of β-galactosidase and the gene i^+ the inducibility state of the enzyme When i^+ is present, the enzyme is actively present if an inducer is present (in the older vocabulary, the enzyme is an adaptive enzyme) When i^- is present, the presence of an inducer is not necessary provided z^+ is also present The enzyme is then constitutive The dominant question which has long existed with respect to induction of enzymes such as β-galactosidase has been, is the inducer doing anything positive in starting synthesis of the enzyme or is it merely relieving an inhibition (as for example by a repressor such as discussed above)? If the inducer is necessary for enzyme synthesis under all conditions, it is required that strains which synthesize β-galactosidase without the addition of exogenous inducer (the constitutive mutants) have a built-in inducer, while the adaptive mutants do not On the other hand, if the inducer is inhibiting the effect of a repressor,

the constitutive mutants have no repressor while the adaptive mutants do

The pajama experiment appears to have resolved this conflict in interpretation By making appropriate conjugations between Hfr and F^-, then preventing the active participation of the Hfr donor after the passage of the relevant genes into the F^- cytoplasm, and determining the β-galactosidase activity of the heteromerozygotes (the fertilized F^- before recombination), it was possible to show that the constitutive (z^+i^-) mutants have no repressor while the adaptive ($z^+ i^+$) strains do The inducibility gene i^+ is dominant over noninducibility i^- Hence, if the cross ♂ (z^+i^+) × ♀ (z^-i^-) is carried out, the ♀ exconjugants before recombinaton are able to start immediate synthesis of β-galactosidase but continue to do so for only a short period of time, after which i^+ exerts its dominance by forming repressor Enzyme synthesis can be started again only by the introduction of outside inducer The conclusion, then, is that inducers are inhibitors of repressors.

The repressor substance produced in the presence of i^+ does not seem to be a protein, since it is apparently actively synthesized in the presence of 5-methyltryptophan It was suggested that the substance may be a nucleic acid or a derivative such as a nucleotide

After a prolonged discussion concerning the role and formation of ribosomes (and their possible transfer from the Hfr to the F^- cell), in which no definite conclusions were arrived at, the discussion of regulation was terminated

Dr Marks then discussed his recent work on glucose-6-phosphate dehydrogenase (G-6-P D) levels in individuals predisposed to acute hemolytic anemia This is an inherited trait, and the anemia can be triggered by primaquine and a number of other substances and factors There is a high incidence of the trait in Kurdistan Jews, Negroes, Caucasians of Greek and Sardinian extraction, and certain South American Indian tribes

Individuals with this trait all show a low level of G-6-P. D activity in their erythrocytes and leukocytes The gene causing it may be sex-linked and somewhat variable in expression with respect to G-6-P. D. activity As might be expected, males have a somewhat lower enzyme activity level than females Caucasian males have a significantly lower level than Negro males Affected females of both racial groups have the same low level, however.

G-6-P D from affected and normal Negro males has been purified and found to require TPN as a coenzyme, both for activity *and as a stabilizer of activity*. Michaelis constants for TPN and G-6-P D

are not significantly different for enzymes from affected and normal individuals Enzyme from both sources also seems to be the same with respect to thermostability, inhibition by various inhibitors, pH optima, and electrophoretic mobility. There is no evidence, therefore, that the enzyme in the affected individuals is altered qualitatively. It is suggested that possibly the mutation causes a reduction in the availability of TPN so that the apoenzyme in afflicted individuals is less stable than in normal individuals This reduced stability is reflected in its lowered activity in the blood cells.

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